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Escherichia coli 30S ribosomal subunit assembly: a novel role for the DnaK chaperone system

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***Escherichia coli* 30S ribosomal subunit assembly:**

A novel role for the DnaK chaperone system

by

Jennifer Anne Maki

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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has met the dissertation requirements of Iowa State University**

Signature was redacted for privacy.

Major Professor

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For the Major Program

This work is dedicated to Eric M. Menor.

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CHAPTER 1. INTRODUCTION

General Introduction

In the late 1930's, Albert Claude first observed ribosomes by dark field microscopy of cell homogenates, and referred to them as "microsomes." Microsomes were initially believed by some to be mere artifacts of cell disruption. In the 1950's, George Palade observed ribosomes by electron microscopy of intact cells, disproving this theory. The ribosome was postulated to be the site of protein synthesis in the cell. This was shown to be true in 1955 by Paul Zamecnik who observed ^{14}C labeled amino acids' fleeting association with ribosomes prior to their appearance in free proteins.

It is now known that prokaryotic ribosomes are large macromolecular particles composed of 2/3 RNA (over 4000 nucleotides) and 1/3 protein (over 50 distinct proteins). The entire ribosome has a sedimentation coefficient of 70S, and it is comprised of two asymmetric subunits, the 50S (large) and 30S (small). S denotes Svedberg units, which are a measurement of the rate at which particles sediment when spun at high speed in an ultracentrifuge. Svedberg units are determined by the size and shape of the particle. The 30S subunit is the site of mRNA and tRNA binding and plays a role in translation initiation and fidelity. It consists of 16S rRNA and 21 proteins. The 50S subunit contains two RNAs, the 5S and 23S rRNA in addition to 34 proteins. The 50S subunit is responsible for peptide bond formation and has also been shown to interact with tRNA.

Although spectacular advances have been made in the structural understanding of the ribosome, relatively little is known about the mechanism by which ribosomes are assembled *in vivo*. To this end, I have employed the recombinant protein system (Culver and Noller 1999) for the production and purification of each individual small subunit protein. Utilizing natural 16S rRNA and the recombinant proteins, I have spent the last four years gaining a better understanding of *in vitro* 30S subunit assembly and applying this knowledge to the *in*

vivo pathway. In experiments using S100, a post-ribosomal *E. coli* extract, I identified the DnaK chaperone as a putative extra-ribosomal assembly factor. In this essay I summarize the first evidence clearly and directly linking the DnaK chaperone system and *E. coli* small ribosomal subunit assembly.

Dissertation organization

This dissertation consists of five main sections. Chapter 1 includes a general introduction, explanation of organization, and literature reviews of ribosome assembly and the DnaK chaperone system.

Chapter 2 is my first paper, "The DnaK chaperone system facilitates 30S ribosomal subunit assembly." In this paper we demonstrate the role of the DnaK chaperone system in small subunit assembly *in vitro* and a possible link between DnaK and ribosome assembly *in vivo*. Some of the techniques utilized include sucrose gradient sedimentation, Western blot analysis, SDS-PAGE, affinity chromatography, tRNA binding, and polysome profile analysis. The second author, Daniel J. Schnobrich, is responsible for production of the data depicted in figure 5 of the text. He performed dilution plating and polysome profiles of the *dnaK756* strains. The remainder of the research is my work, and the text and figures were prepared by me and Dr. Culver. The paper was published in Molecular Cell (Maki et al., 2002).

Chapter 3, "Demonstration of the role of the DnaK chaperone system in assembly of 30S ribosomal subunits using a purified *in vitro* system," is the second publication from my research here at Iowa State University. This is a response to a paper that challenged the data from our first publication. Defined system polyphenylalanine synthesis was used to show that *in vitro* assembled subunits formed at low temperature with the DnaK chaperone system are functional. Daniel R. Southworth contributed to this work by performing the defined polyphenylalanine synthesis assay developed in the Green lab (Southworth et al., 2002) and

by preparing figure 3. The remainder of the research, figures 1 and 2, and all of the text were generated by Dr. Culver and me. The manuscript was published in RNA (Maki et al., 2003).

“Association of the DnaK chaperone system components with *Escherichia coli* small ribosomal subunit intermediates” is Chapter 4, my third and final manuscript. The DnaK chaperone system components were found to interact with the assembly intermediates in a manner similar to their well-studied interaction with protein substrates. Additionally, DnaK bound 16S rRNA and pre-16S rRNA along with S4 (and to a lesser extent, S3) *in vivo*. Techniques employed include sucrose gradient sedimentation, semi-quantitative Western blot analysis, affinity chromatography, primer extension and stability assays. The manuscript was submitted to the Journal of Molecular Biology on June 24, 2004.

Finally, in Chapter 5, general conclusions about my research career are made. Broad implications of this work are outlined, and future directions will also be explored.

Literature review

In the last forty years, the work addressing the protein synthesizing machinery of the cell has seen much progress. In this cursory overview of the field, several topics will be addressed. The first and most thorough discussion will detail several years of research by Masayasu Nomura, the father of *in vitro* 30S ribosomal subunit reconstitution. His laboratory’s detailed analysis of this process has made my course of study possible. The second topic in the literature overview is the development of the recombinant protein system for 30S ribosomal subunit assembly *in vitro* in Harry Noller’s laboratory, another advance crucial to my work. The structural studies of the ribosome will be briefly touched on, followed by a glimpse of extra-ribosomal assembly factors identified thus far in *E. coli*. Since my research has linked ribosome biogenesis with the DnaK chaperone system *in vitro* and *in vivo*, the final section of the literature review will be a concise background of this chaperone system’s components and how they work together.

Early in vitro reconstitution advances by Masayasu Nomura

After three years as faculty at Osaka University in Japan, Masayasu Nomura accepted a faculty position at the University of Wisconsin Madison in the 1960's. He began his study of *E. coli* ribosomes there and became a crucial contributor to the field. Nomura's laboratory found that when *E. coli* ribosomes were spun on cesium chloride gradients in the ultracentrifuge, a group of proteins was stripped from the 30S subunit. They named the remaining ribonucleoprotein particle (RNP) "core particle," and the proteins in this particle were termed CP30 (core particle proteins from 30S). The proteins that split from this core particle were referred to as SP30 (split proteins from 30S). When 16S rRNA and CP30 were incubated at 37°C, then cooled and incubated at low temperature with SP30, functional 30S subunits were formed. This was only the first of many reconstitution experiments conducted in the Nomura laboratory. In a breakthrough experiment, they demonstrated that 16S rRNA and Total Proteins from the 30S subunit (TP30) could be incubated together at 37°C to form 30S subunits. This was an important step because it revealed that assembly could occur when all of the individual components of the 30S subunit were mixed. From these results, they concluded that the information for assembly of 30S subunits is contained within its components, and *in vitro* assembly of 30S ribosomal subunits was born (Traub and Nomura, 1968).

Next, work was done to determine the optimum *in vitro* reconstitution conditions. They found the ideal ionic strength for reconstitution was 0.37, and postulated the necessity for such a high ionic strength is to avoid non-specific aggregation, since RNA is negatively charged, while small subunit ribosomal proteins are positively charged. Therefore, the ionic strength of 0.37 ensures that only strong, specific and hence productive interactions between RNA and proteins will occur. Additionally, Nomura's lab found the Mg^{++} concentration needs to be at least 10 mM to hold the 30S subunit together. Finally, the ideal reconstitution

pH value was a less stringent requirement; optimum reconstitution was achieved between 6.5 and 8.0 at 40°C. They also determined that the assembly of 30S subunits is a first-order reaction with an activation energy of 38 kcal/mol (Traub and Nomura, 1969).

At low temperature, 16S rRNA and TP30 yield a non-functional assembly intermediate termed Reconstitution Intermediate (RI) that sediments at 21S. RI has only a subset of the small ribosomal subunit proteins bound. When RI is heat-treated, a conformational change occurs and an activated particle (RI*) is formed. RI* is able to bind the remaining ribosomal proteins, even at low temperature, to form a functional 30S subunit. Traub and Nomura recognized that the RI to RI* transition is the rate-limiting step in assembly and therefore has a high activation barrier, accounting for the activation energy of 38 kcal/mol for the process of 30S subunit assembly (Traub and Nomura, 1969).

At this point, great progress had been made in the study of *in vitro* small subunit assembly, but a connection between *in vitro* results and the actual *in vivo* system would demonstrate the importance of the work done in the Nomura lab. Since the rate-determining step of *in vitro* 30S subunit assembly requires heat, they postulated that mutants with assembly defects would have pronounced phenotypes at low temperature. This theory was verified by the identification of ribosomal subunit assembly intermediates accumulating in mutants grown at low temperature. Nomura and colleagues studied *E. coli* mutants that fail to grow at 20°C, termed subunit assembly defective, or sad mutants. The cold sensitive mutants were grown at 25-30°C, and their cellular extracts were studied via analytical ultracentrifugation. Among these cold sensitive mutants, three general types were observed. The first two types were defective in 50S subunit biosynthesis. They accumulated 43S or 32S particles, both being 50S precursors that contained 23S rRNA. The third type of mutant produced less 50S subunits and accumulated 21S particles, precursors to 30S subunits containing 16S rRNA (Guthrie et al., 1969).

Another cold sensitive mutant used to study RI particles was *spc-49-1*. This was a spectinomycin resistant mutant from *E. coli* N049. At low temperature, a 21S precursor particle accumulated, and the researchers assessed it in two different ways. First, the proteins of the 21S particle were analyzed and shown to correspond to the early assembly proteins in 30S subunit assembly. Next, in a very elegant experiment, the cells from *spc-49-1* were ^3H uracil labeled at low temperature, and the culture was split into two fractions. From the first fraction, an extract was immediately prepared and analyzed using analytical ultracentrifugation. ^3H labeled 21S particles and 30S subunits were both present in this extract. The second portion of cells were pelleted, washed, and finally incubated with non-radioactive uracil at 42°C. The extract prepared from these cells contained only ^3H labeled 30S subunits. The radioactive 21S particles were gone, having been completely converted into 30S subunits. The formation of 30S subunits from *in vivo* 21S particles demonstrates that 21S is a true assembly intermediate, not a degradation product of 30S subunits (Nashimoto et al., 1971). Two independent laboratories confirmed these findings. In 1973, Nierhaus and coworkers found an RI-like particle that they termed p30S. Analysis by two-dimensional gel electrophoresis showed p30S had a similar protein complement to RI (Nierhaus et al., 1973). Another such precursor particle was identified *in vivo* via pulse-labeling experiments, and 16S rRNA was observed as a component of the precursor (Lindahl et al., 1975), again demonstrating that RI is a true small subunit assembly precursor, and the assembly pathway observed *in vitro* is likely the same as the *in vivo* path.

Next, Nomura's lab individually purified the small subunit ribosomal proteins from *E. coli* via column chromatography so they could dissect the pathway of assembly. With each individual protein at their disposal, Nomura and his students began the painstaking process of determining the order in which the ribosomal proteins bind the growing small subunit. Initially, each protein was incubated with 16S rRNA and the interaction between

protein and RNA (if any) was observed. In this manner, they determined which proteins were capable of binding naked 16S rRNA (these proteins are now termed primary binding proteins). Once a primary binding protein was identified, the 16S rRNA and single primary binding protein complex was formed and used to screen for interactions with the remaining small subunit proteins. In this manner, a single branch of the assembly map was worked out. Through numerous experiments, the framework of the entire assembly map emerged (Mizushima and Nomura, 1970).

Subsequently, single protein omission studies were initiated. When one small subunit ribosomal protein was left out of the reconstitution, what was the effect on sedimentation and/or function of the particle that was formed? Predictably, omission of a primary binding protein caused the most drastic effect. Particles resulting from single omission studies were also analyzed for protein composition. They were sucrose gradient purified, the fractions from the gradient were pooled and centrifuged to concentrate, and the particles were then subjected to PAGE. Densitometer tracings from the gel gave an indication of the dependence of individual proteins on one another in the assembly pathway (Mizushima and Nomura, 1970). The assembly map underwent several iterations, and several proposed interactions are still under investigation, but in 1974 a map very similar to the one used today emerged (Held et al., 1974).

The assembly map (Figure 1, page 48) represents the order of interactions in the process of 30S subunit assembly. The 16S rRNA is represented as a rectangle. Primary binding proteins (S4, 7, 8, 15, 17, 20) are shown in black and are able to bind naked 16S rRNA independently. Secondary binding proteins (S5, 6, 11, 12, 13, 16, 18, 19) are colored purple and require the prior association of at least one primary binding protein before they associate with the particle. Tertiary binding proteins (S2, 3, 10, 14, 21), shown in blue, depend on at least one primary and one secondary binding protein for their association with

the growing RNP. The dashed line in the assembly map divides the tertiary binding proteins from the RI particle, which consists of 16S rRNA, primary and secondary binding proteins.

A recombinant protein system for in vitro 30S subunit assembly

Although the scientific groundwork for 30S subunit assembly had been laid, the *in vitro* reconstitution process was still highly inefficient using the individually purified proteins, and thus difficult to study. In her postdoctoral work in the laboratory of Dr. Harry Noller at UC-Santa Cruz, Dr. Gloria Culver addressed this problem. She reasoned that if each of the small ribosomal subunit proteins was cloned and overexpressed, more protein could be produced with higher purity and in a more cost-efficient manner. The DNA for each of the small subunit proteins was cloned into the pET24b vector, which has an IPTG inducible promoter. When this vector was transformed into *E. coli* strain BL21, protein overexpression was made possible. The individual recombinant proteins were purified via FPLC under different conditions according to their individual properties (Culver and Noller, 1999).

Once individually purified recombinant proteins were produced, the order of assembly was studied. It had been previously determined that ordered assembly resulted in 45% of input RNA ending up in 30S subunits, whereas only 18% was found in 30S subunits when all of the proteins were added at once (Mizushima and Nomura, 1970; Held et al., 1974). Two methods to ordered assembly were attempted. In the first, the characterizations of early, mid and late assembling proteins from *in vitro* assembly kinetics studies from the Noller lab were used (Powers et al., 1993). The second approach was based on the assembly map from the Nomura lab (Held et al., 1974) and provided the best reconstitution. 30S subunits formed with individually purified recombinant proteins were tested for functional ability via subunit association with natural 50S subunits, tRNA binding and polyphenylalanine synthesis. The recombinant system performed approximately 50% as well

as natural 30S subunits in tRNA binding and 30% as well in polyphenylalanine synthesis. Reconstitution with TP30 is much more efficient than with recombinant proteins, and the values for reconstituted TP30 30S particles were 70% for tRNA binding and 80% for polyphenylalanine synthesis. Although TP30 reconstitutions form the most functional 30S subunits, reconstitutions with recombinant proteins were a vast improvement over those performed with individually purified natural components. Furthermore, the 30S recombinant protein system allows for focused studies manipulating single components to assess the structure and function of 30S subunits (Culver and Noller, 1999).

Structural studies of the small ribosomal subunit

The early focus in the study of ribosome function was on the proteins' role. Kurland suggested that perhaps the RNA plays an even more significant function than the proteins, but his hypotheses were controversial at the time (Kurland, 1974). Noller and Woese helped shift the thinking toward an RNA-based ribosome when they began studying the 16S rRNA rather than the small subunit proteins (Noller and Woese, 1981). It had been previously noted that 16S rRNAs from many bacteria were interchangeable in reconstitution experiments (Nomura et al., 1968), suggesting a high degree of conservation of RNA. In 1992, the importance of RNA was demonstrated when the rRNA from the 50S subunit of a thermophile was used to catalyze peptide bonds, even after almost all of the 50S proteins had been removed (Noller et al., 1992). Using enzymatic data, chemical modification and sequence analysis, Noller and Woese (1981) developed a model for the secondary structure of 16S rRNA. Their results revealed that much of the 16S rRNA was not base paired. In fact, eight consecutive base pairs constituted the longest run of base pairs in a stem of the structure. Thus, much of the 16S rRNA is found in unpaired stem loops, as unpaired bases bulge out from each other (Noller and Woese, 1981). In more recent work by Gutell and coworkers it was determined that many loop regions of RNA are adenosine rich and therefore

adenosines in particular account for many of the unpaired bases. They named these unpaired adenosine regions A-motifs, and postulated that they are crucial in RNA folding from secondary to tertiary structure (Gutell et al., 2000).

The importance of the 16S rRNA in formation of the 30S subunit was further shown in studies that demonstrated the ability of its domains to assemble independently of one another. Transcribed fragments of 16S rRNA were used in conjunction with TP30 to test the ability of the RNA's structural domains to assemble independently. The central domain of 16S rRNA (the portion which folds to become the platform of the 30S subunit) was shown to be capable of such assembly (Agalarov et al., 1998). Next, the 5' and 3' domains, correlating to the body and head of the 30S subunit, respectively, were reconstituted (Agalarov et al., 1999). The 3' domain (the head of the 30S subunit) had already been independently reconstituted in the laboratory of Harry Noller (Samaha et al., 1994). These results taken together demonstrate that the domains of 16S rRNA correspond to distinct portions of the 30S subunit: the head, platform and body. The domains and structural components in turn correspond to the organization of the assembly map, suggesting the ordered assembly that has been observed is due to the independent assembly of each structural domain of 16S rRNA with the appropriate small subunit proteins.

Early structural details of ribosomes were attained utilizing immunoelectron microscopy (Stoffler and Stoffler-Meilicke, 1984). These structures were refined in the 1990s when cryo-electron microscopy was developed and applied to the ribosome. Multiple images were acquired and used together to reconstruct the ribosome structure (Frank et al., 1995). This method, though refined compared to immunoelectron microscopy, still gave a relatively crude picture of ribosome structure. Finally, crystal structures were obtained of the 30S subunit (Wimberly et al., 2000), the 50S subunit (Ban et al., 1999), both the 30S and 50S subunits (Schleunzen et al., 2000; Harms et al., 2001), and the 70S ribosome (Yusupov et al.,

2001). These structures were solved from crystals of *Thermus thermophilus* ribosomes and ribosomal subunits, and allow atomic resolution. Now that the crystal structures have been solved, biochemical data can be verified by structural information, and the structures can be used to design biochemical experiments. This enormous advance should push the field ahead at a very rapid pace. Although the structure allows us to create informed hypotheses and may assist in resolving disputes over incongruent biochemical data, the structure cannot provide us with details of the process of ribosome assembly dynamics.

Extra-ribosomal assembly factors

Although a myriad of factors are known to assist in eukaryotic ribosome assembly (Fromont-Racine et al., 2003), few factors have been implicated in prokaryotic ribosome assembly. Of the examples in prokaryotes, some are concerned with 50S subunit assembly. SrmB, a DEAD-box RNA helicase, has been shown to associate with pre-50S particles in wild type cells. When *srmB* is deleted, aberrant polysome profiles result. The 50S peak is decreased dramatically, and 40S particles accumulate. These 40S particles contain precursor 23S rRNA but are missing several late assembly r-proteins and also L13, an early assembly r-protein (Charollais et al., 2003).

Another factor, CsdA, can be found associated with pre-50S particles in wild type cells. CsdA is another DEAD-box protein and a cold shock RNA helicase known to participate in translation initiation but only required in the cell below 30°C. In the *csdA* deletion strain, 40S particles amass that differ from those found in the Δ *srmB* strain. These particles contain precursor 23S rRNA and do not contain some late assembly proteins. Interestingly, overexpression of CsdA corrects the Δ *srmB* ribosome defect (Charollais et al., 2004).

In the quest for extra-ribosomal assembly factors, 30S subunit assembly factors have also very recently emerged. Some of the initial work was done in 1993 by Alix and

colleagues, who determined that mutations in *dnaK* caused ribosome assembly defects in *E. coli*. *dnaK756*, a temperature sensitive mutant (Georgopoulos et al., 1979), accumulates ribosomal precursor particles at non-permissive temperature (45°C). The precursors sediment at 25S, 35S and 45S and are converted to ribosomal subunits once shifted to permissive temperature (30°C). The 25S particles contained 16S rRNA, the 45S contained 23S rRNA, and the 35S particles contained both (Alix and Guerin, 1993).

In later work, Alix and colleagues claimed, “DnaK acts during ribosome assembly itself, and not by stabilizing mature ribosomes or protecting them from thermal injury (Hage et al., 2001).” In this same body of work, it was determined that DnaJ was also required for ribosome assembly, suggesting that the DnaK chaperone system may work via its protein folding pathway (Bukau and Horwich, 1998). The authors also found that GroEL was necessary at 45°C for the last step (45S→50S) of large subunit assembly (Hage et al., 2001).

At this point, Alix’s group proposed two hypotheses concerning the way DnaK and GroEL function in ribosome assembly above 37°C. In the first, “late” assembling r-proteins are assisted in folding onto the growing RNP by the DnaK and GroEL systems. This function would simply represent one more interaction DnaK has with polypeptide chains. The second hypothesis was that DnaK and GroEL assist in assembly by interacting with ribosomal RNA, perhaps adjusting its conformation to allow complete assembly. This hypothesis would stretch the known capabilities of the DnaK chaperone system into a new arena. Whatever the mechanism, Alix’s laboratory hoped to eventually reconstitute ribosomes *in vitro* using chaperones at conditions of lower temperature, shorter incubation times, and more physiological salt concentrations (Hage et al., 2001), unlike *in vitro* reconstitution without the assistance of extra-ribosomal assembly factors (Traub and Nomura, 1969; Culver and Noller, 1999).

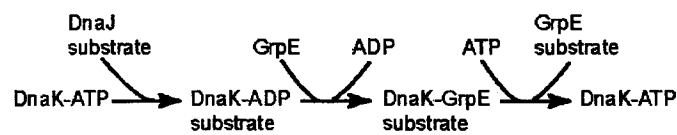
In the summer of 2000, I began my Ph.D. research in the laboratory of Dr. Gloria Culver. Utilizing the recombinant protein system developed for 30S subunit assembly (Culver and Noller, 1999), I began the search for extra-ribosomal assembly factors. Dr. Culver had previously observed that low temperature reconstitutions of recombinant small subunit proteins with natural 16S rRNA produced 30S subunits when S100 extract, a crude post-ribosomal *E. coli* extract, was included in the reconstitution (Culver, unpublished results). My first objective was to isolate and characterize the unknown assembly factor(s) in S100 responsible for the conversion of RNPs from 21S to 30S at low temperature. A non-ribosomal protein was found associated specifically in such low temperature reconstitution experiments, and N-terminal sequencing revealed its identity as DnaK (Maki et al., 2002). Although data from Alix and colleagues had been suggestive of a link, this was the first direct connection demonstrated between DnaK and 30S ribosomal subunit assembly.

Once DnaK was identified associated with the assembling 30S subunit out of S100 extract, we decided to further investigate its involvement in assembly. The purified DnaK chaperone system was shown to facilitate 30S subunit assembly *in vitro* at otherwise non-permissive temperature. Particles formed in this manner co-sediment with and have the same protein complement as 30S subunits. Additionally, they are functional in tRNA binding (Maki et al., 2002) and polyphenylalanine synthesis (Maki et al., 2003). Furthermore, the association of DnaK chaperone system components with the assembling 30S subunit is similar to their association with protein substrates (Maki and Culver 2004). Also, DnaK associates with 16S and pre-16S rRNA and small subunit protein S4 *in vivo*, suggesting a role for DnaK in early assembly of the 30S subunit (Maki and Culver 2004).

The DnaK chaperone system

DnaK's most well-characterized role is in protein folding (for review see Bukau and Horwich, 1998; Hartl and Hayer-Hartl, 2002). It is an hsp70 (heat shock protein, 70 kDa)

chaperone. Chaperones allow proper folding of proteins by protecting exposed hydrophobic patches (a hallmark of unfolded proteins) from aggregation. There are three main categories of protein folding chaperones. Trigger factor (TF) is a ribosome-bound protein folding machine (Hesterkamp et al., 1997), the DnaK chaperone system primarily functions by folding proteins in the cytosol (see following), and the GroEL system (a chaperonin) folds proteins by sequestering them within its cylindrical chamber (Walter, 2002). These protein folding systems are conserved in Eubacteria, Archaea and Eukarya (Hartl and Hayer-Hartl, 2002). Chaperones and chaperonins assist many proteins in attaining a functional fold. 65-80% of proteins in the cell fold by interaction with TF, 10-20% by the DnaK chaperone system, and 10-15% require interaction with the chaperonin GroEL (Hartl and Hayer-Hartl, 2002).



The DnaK chaperone system is comprised of DnaK, its co-chaperones DnaJ and GrpE, and requires ATP for its functional cycle (depicted above). DnaK is generally accepted as a chaperone found free in the cytosol, although it has also been observed bound to ribosomes (Ghosh et al., 2003; Vysokanov, 1995). DnaK is a 70 kDa protein with an N-terminal ATPase domain (residues 1-385), a central substrate binding domain (residues 385-561) and a C-terminal domain (561-638) of unknown function (Mayer et al., 2000). Both the nucleotide and substrate binding sites of DnaK are conformationally affected by the binding of either nucleotide or polypeptide (Fink, 1999; Han and Christen, 2003). The DnaK chaperone system protein folding cycle is dependent on DnaJ's ability to recruit substrate and stimulate ATP hydrolysis by DnaK. DnaK binds substrate tightly once in the ADP state. GrpE binds DnaK's ATPase domain and effects nucleotide exchange and concomitant

substrate release. This brings DnaK back to its low affinity ATP bound state, and the folding cycle begins anew (Bukau and Horwich, 1998).

DnaK is present in the cell at a concentration of 50 μM , while ribosomes are present at a concentration of 30 μM (Teter et al., 1999). It has a low inherent ATPase activity to guard against futile ATP consumption (Han and Christen, 2001). The crystal structure of the substrate binding cavity of DnaK has been solved. It has a central hydrophobic pocket for binding substrate in addition to an arch and helical lid to close over the substrate once bound, and ATP assists in the regulation of substrate binding through DnaK's β domain. All of these structural components contribute to DnaK's affinity for substrate (Mayer et al., 2000). DnaK binds only L-amino acids, and the backbone orientation of substrate is crucial to binding (Rudiger et al., 2001).

DnaJ is a member of the hsp40 family of proteins. These proteins contain an N-terminal J domain that interacts with DnaK to stimulate its ATPase activity. DnaJ also contains a Zn finger domain in its central region where it binds proteins. The domains found between the J domain and Zn finger domain and at the C-terminal end of DnaJ have unknown function (Fink, 1999). DnaJ is a chaperone in its own right, as it binds hydrophobic patches of proteins. Its substrates are similar to those of DnaK, and this is how it recruits substrate to DnaK. DnaJ binds stretches of 8 hydrophobic residues, favoring aromatic and hydrophobic aliphatic residues. However, unlike DnaK, DnaJ's binding is due to side chain contacts and is not stable (Rudiger et al., 2001). This could aid in DnaJ's function of recruiting substrate but then delivering it to DnaK, a chaperone that is capable of binding the substrate more tightly.

GrpE binds DnaK's ATPase domain as a dimer. It has two long α helices (residues 40-88) that curve toward DnaK. GrpE also contains a central four helix bundle (residues 89-137) and C-terminal compact β domains (residues 139-197) (Harrison et al., 1997). GrpE's

role in the DnaK chaperone cycle is to induce the release of ADP from DnaK. As such, it is referred to as a nucleotide exchange factor for DnaK. Once ATP re-binds DnaK, the DnaK peptide complex dissociates (Hartl and Hayer-Hartl, 2002). GrpE has also been designated as a thermosensor for the cell because its nucleotide exchange activity is temperature sensitive. This is likely due to the unfolding (“melting”) of the long paired helices at and above 50°C. When the helices become unfolded, nucleotide exchange on DnaK does not occur. At very high temperatures, this could be the last effort to save the cell by allowing DnaK to sequester as much unfolded protein as possible, preventing massive aggregation (Gelinas et al., 2002).

Although the DnaK chaperone system plays an important role in the cell, DnaK is only essential above 37°C and below 15°C. Of the other two main protein folding systems, trigger factor (TF) and GroEL/ES, only GroEL/ES is essential at all temperatures, whereas TF is not essential at any temperature. However, a double deletion strain of TF and DnaK, $\Delta tig\Delta dnaK$, is a synthetic lethal at 30° and 37°C (Deuerling et al., 1999; Teter et al., 1999). Very recently it was shown that such a double deletion mutant is viable at temperatures less than 30°C or with the overproduction of the GroEL/ES system at 30°C. No other chaperone could be substituted in this situation, and the authors surmise this is due to GroEL/ES working as a folder protein rather than a holder. That is, GroEL/ES actively folds proteins, whereas holder proteins merely prevent aggregation (Vorderwulbecke et al., 2004).

Although DnaK’s role in protein folding is currently its best characterized, other functions for DnaK and other chaperones have also come to the forefront recently. Many of these functions involve the binding of RNA. Hsp70 has been shown to interact with 5S rRNA in a complex that was separated from the ribosome with high salt treatment (Okada et al., 2000). Zimmer and colleagues found that DnaK binds RNA in a sequence-specific manner. This binding occurs in the N-terminal ATPase of DnaK, and AUUUA motifs were

vital to this DnaK-RNA interaction (Zimmer et al., 2001). Validating this result, mammalian hsp70 has been found in complex with A + U rich elements (AREs), and could therefore be involved in ARE-directed mRNA turnover (Wilson et al., 2001). Additionally, hsp70 homologues have been found bound to polysomes *in vivo* (Beck and DeMaio, 1994; Pfund et al., 1998) which could also be due to DnaK's ability to bind RNA.

GroEL, the 60 kDa chaperonin, has been identified as a part of the protection complex for mRNA (Georgellis et al., 1995). GroEL has also been shown to co-purify with RNase E activity. It has been speculated that 9S rRNA processing is regulated by the GroEL-RNase E interaction (Sohlberg et al., 1993). In a most intriguing study, the 60 kDa chaperonin from *Sulfolobus solfataricus* was found associated with pre-16S rRNA with an unprocessed 5' end. Primary binding proteins were also part of the complex, and it was postulated that the chaperonin binds the 16S rRNA and aids in its cleavage (Ruggero et al., 1998).

Finally, a small heat shock protein, hsp15, has been shown to bind the ribosome. The crystal structure of hsp15 reveals an RNA binding motif like that found in small ribosome subunit protein S4. Although the sequence homology is low, the α L motif structure is very similar in these two proteins (Staker et al., 2000).

Clearly, this is only the beginning of chaperones and chaperonins in non-protein binding roles. These helper proteins are turning up in studies across phylogeny, and it is a very exhilarating time to be studying DnaK in such a role.

Conclusions

The field of 30S ribosomal subunit assembly has seen great progress in the last forty years, but despite the biochemical work of many different research groups and the recently solved crystal structures (Ban et al., 1999; Wimberly et al., 2000; Schleunzen et al., 2000; Harms et al., 2001; Yusupov et al., 2001), there is still much to be learned concerning the

details of how this large RNP is assembled. The observation that S100 extract can potentiate the formation of 30S subunits *in vitro* was a very exciting first step in elucidating how assembly occurs. The discovery that the DnaK chaperone system is sufficient to effect this formation has opened a new course of investigation concerning 30S subunit ribosome assembly. The link between ribosome assembly and the DnaK chaperone system is explored in this dissertation.

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CHAPTER 2. THE DNAK CHAPERONE SYSTEM FACILITATES 30S RIBOSOMAL SUBUNIT ASSEMBLY

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Running Title: Assembly of the *E. coli* 30S ribosomal subunit

Abstract

Functional *Escherichia coli* 30S ribosomal subunits can be reconstituted in vitro. However, slow kinetics and sharp temperature dependence suggest additional assembly factors are present in vivo. Extract activation of in vitro assembly results in association of DnaK/hsp70 chaperone components with pre-30S particles. Purified DnaK, its cochaperones DnaJ and GrpE, and ATP can facilitate reconstitution of functional 30S subunits under otherwise nonpermissive conditions. A link has been observed between DnaK, 30S subunit components, and ribosome biogenesis in vivo as well as in vitro. These studies reveal a novel role for the DnaK/hsp70 chaperone system, in addition to its well-documented role in protein folding, and suggest that 30S subunit assembly can be facilitated.

Introduction

Ribosomes are ubiquitous ribonucleoprotein particles (RNPs) that are responsible for the fundamental process of protein synthesis. *Escherichia coli* (*E. coli*) 70S ribosomes are

composed of two asymmetric subunits, designated small and large. The 30S subunit, which is comprised of one RNA (16S rRNA; 1542 nucleotides) and twenty-one unique proteins (S1-S21), is compositionally less complex than its larger 50S counterpart. The three major domains of 16S rRNA (Noller and Woese, 1981), with their associated proteins, are capable of assembling independently of one another and can form autonomous structures (Weitzmann et al., 1993; Samaha et al., 1994; Agalarov et al., 1998; Agalarov et al., 1999; Ramakrishnan and Moore, 2001). Such autonomous structures have not been demonstrated for the 50S subunit and the intertwined domains revealed in structures of 50S subunits [see Ramakrishnan and Moore, 2001 for review] suggest that these structures may not exist for this subunit. Given these observations, it follows that many studies of ribosome assembly have focused on the 30S subunit or domains of this subunit.

Many 30S subunit assembly experiments have taken advantage of the ability of these particles to be reconstituted *in vitro* using purified components. *In vitro* reconstitution of functional 30S subunits can be supported using a mixture of Total Proteins isolated from the 30S subunit (TP30), individually purified natural (Held et al., 1973) or recombinant (Culver and Noller, 1999) small subunit proteins and 16S rRNA. The interdependence of ribosomal protein association with 16S rRNA and various RNPs during 30S subunit formation was determined using these systems (Mizushima and Nomura, 1970; Held et al., 1974; Fig. 1). Some of the small subunit proteins, the primary binding proteins (1^o; Fig. 1A black) can bind independently and specifically to naked 16S rRNA. The secondary binding proteins (2^o; Fig. 1A pink) require the prior association of at least one primary binding protein before they are able to interact appropriately with the growing RNP. Finally, the tertiary binding proteins (3^o; Fig. 1A blue) bind once primary and secondary binding proteins have associated to complete the cooperative assembly of functional 30S subunits.

In vitro 30S subunit assembly is characterized by slow kinetics and is dependent upon optimal temperature and ionic conditions. At low temperatures, where *E. coli* growth is uncompromised, reconstitution stalls and a particle which sediments at 21S is formed (Traub and Nomura, 1968; Fig. 1B). The 21S Reconstitution Intermediate (RI) contains a subset of the small subunit ribosomal proteins, corresponding to the primary and secondary proteins, and 16S rRNA (Held and Nomura, 1973; Fig. 1BI). A temperature-dependent conformational change is required to convert RI to an assembly-competent intermediate, RI* (here denoted Δ RI*), which sediments at 26S (Fig. 1BII). Once Δ RI* is formed, it is competent for assembly of the tertiary binding proteins (Fig. 1B III). Interestingly, an intermediate similar to RI has also been observed *in vivo* (Guthrie et al., 1969; Nashimoto et al., 1971; Nierhaus et al., 1973; Lindahl, 1975; Alix and Guerin, 1993), strongly suggesting that *in vitro* and *in vivo* 30S subunit assembly follow similar paths. The observed *in vitro* and *in vivo* intermediates and the characteristics of *in vitro* 30S subunit reconstitution suggest that factors which normally assist 30S subunit assembly *in vivo* are lacking *in vitro*. Here, data are presented to support the existence of such factors.

Results

Partially purified *E. coli* extract facilitates *in vitro* reconstitution of 30S ribosomal subunits.

In this study, the temperature-sensitive nature of *in vitro* 30S subunit reconstitution was used to identify factors that can potentiate 30S subunit assembly at low temperature. Reconstitutions performed at low temperature (15°C) using 16S rRNA and either TP30 (data not shown) or the complete set of recombinant proteins (all; Fig. 2Aa), result in particles that sediment near 21S. As expected, when the same reaction is heated to 42°C (designated by Δ) 30S subunits are formed (Fig. 2Ae; also see Fig. 3Ad). Incubation of partially purified *E. coli* extract (S100) with RI at 15°C results in appearance of particles that co-sediment with

30S subunits (Fig. 2A, compare b with a and e). The conversion of RI to 30S particles occurs in an S100 dose-dependent manner (compare Fig. 2A, c with d). These results suggest that some component(s) of the extract is responsible for and capable of generating 30S subunits.

***E. coli* DnaK/hsp70 chaperone system proteins associate with pre-30S subunit intermediates.**

Analysis of the proteins associated with the extract treated reconstitution mixtures revealed two non-ribosomal proteins co-sedimenting with the assembly intermediates (Fig. 2B, lane 1 arrows a and b). Sedimentation of S100 extract alone followed by analysis of the appropriate region of the gradient revealed that one of these proteins (Fig. 2B, arrow b) is a contaminant that fortuitously co-sediments with the intermediates. However, the protein indicated by arrow a (Fig. 2B lane 1) is present only when 30S subunit intermediates (not fully formed 30S subunits; data not shown) were treated with extract. This suggests that this protein specifically associates with assembly intermediates and is therefore a likely candidate for a 30S subunit assembly factor.

N-terminal protein sequence analysis (five amino acids) of this RI-associated protein revealed a complete match to the N-terminus of DnaK, a 70 kDa *E. coli* heat shock protein (hsp70). DnaK is a molecular chaperone that generally acts to influence protein folding [see Bukau and Horwich, 1998 and Agashe and Hartl, 2000 for reviews]. Western blot analysis using an anti-DnaK antibody verified that the intermediate-associated protein was DnaK, and that DnaK could associate with the intermediate either in the presence or absence of tertiary binding proteins (Fig. 2CI lanes 6 and 7; note: no attempt has been made to quantify these results.) No DnaK was found associated with particles in the absence of extract treatment (Fig. 2CI lanes 3-5). These results indicate that DnaK can associate with pre-30S subunit intermediates in the context of a relatively crude cellular extract. This, taken together with

the activation of 30S subunit assembly by extract treatment, suggests that DnaK could be an extract component involved in facilitating 30S subunit assembly.

Given the association of DnaK with the 30S subunit assembly intermediates, we wished to determine if the DnaK co-chaperones GrpE and DnaJ (together with DnaK: DnaK/hsp70 chaperone system) were also associated with these particles. GrpE is a nucleotide exchange factor for DnaK which replaces bound ADP for ATP while likely altering the conformation of DnaK (Liberek et al., 1991; Dekker and Pfanner, 1997). Since GrpE (24 kDa) is approximately the same molecular weight as some of the small subunit proteins, it was difficult to directly identify it as a component of the isolated intermediates. Western blot analysis revealed that GrpE associates with pre-30S subunit intermediates that had been treated with extract (Fig. 2CII lanes 6 and 7). No GrpE was detected in the absence of extract treatment (Fig. 2CII lanes 3-5). Thus, out of the extract both DnaK and GrpE can stably associate with pre-30S subunit assembly particles. DnaJ (40 kDa), which normally acts to stimulate substrate binding and the ATPase activity of DnaK (Wall et al., 1994), was not found associated with extract treated pre-30S particles, although it could be readily detected in the extract using an anti-DnaJ antibody (data not shown). Nevertheless, DnaJ might participate in 30S subunit assembly via a transient interaction, consistent with its proposed action in protein folding (see Fink, 1999 for review). This type of interaction would not be detected in our current analysis.

Purified DnaK/hsp70 chaperone system components can facilitate 30S subunit reconstitution at low temperature.

To determine if the DnaK/hsp70 chaperone system participates in 30S subunit assembly, *in vitro* reconstitution experiments were performed using purified DnaK, GrpE, and DnaJ. At low temperature where *in vitro* reconstitution of 30S subunits is stalled (see Fig. 1B), DnaK, GrpE, DnaJ and ATP can activate the conversion of RI (Fig. 3Aa) to 30S

particles (Fig. 3A, compare b with a and d). Use of a slowly hydrolyzable ATP analog (ATP- γ -S) in place of ATP inhibits conversion of RI to 30S particles (Fig. 3Ac). The requirement for DnaJ and ATP hydrolysis are consistent with the known role of DnaJ in accelerating the rates of ATP hydrolysis and substrate binding by DnaK (Liberek et al., 1991; Wall et al., 1994). These results indicate that DnaK, DnaJ, and GrpE, in concert with ATP hydrolysis, facilitate 30S subunit assembly under otherwise non-permissive conditions. While the 25 unique constituents used in these studies are each individually purified to near homogeneity (>90% pure; see Culver and Noller, 2000 and Materials and Methods), the formal possibility remains that a minor contaminant also contributes to the activity. Such a component could possibly interact with a chaperone or small subunit protein and therefore by definition would be part of the 30S subunit assembly system.

Chaperone-assembled 30S subunits are authentic.

To confirm that the chaperone-assembled particles were indeed 30S subunits, the association of the tertiary binding proteins, which only occurs after the RI to RI* transition, was monitored. Since S2 and S3 are tertiary binding proteins and are two of the largest small subunit proteins, we could readily monitor their association with the different RNPs by SDS-PAGE. As expected, S2 and S3 are associated with 30S particles formed by heat activation (Fig. 3B lane 3) as well as with those formed at low temperature in the presence of DnaK, DnaJ, GrpE, and ATP (Fig. 3B lane 4). The primary binding protein S4 and a collection of smaller, less well resolved 30S subunit proteins are present in Δ RI* (Fig. 3B lane 2) and in the chaperone and heat activated 30S subunits (Fig. 3B lanes 3 and 4). Since RI is relatively unstable, (Held and Nomura, 1973) no proteins are detected from this purified particle (Fig. 3B lane 1), suggesting the particles are dissociating during recovery (see Materials and Methods). These results indicate that the DnaK chaperone system assembled particles are authentic 30S subunits with the tertiary binding proteins associated.

The functional state of the 30S subunits produced using the DnaK chaperone system was assessed using transfer RNA (tRNA) binding (see Nomura et al., 1969; Culver and Noller, 2000), a standard assay for monitoring the functional capacity of reconstituted 30S subunits. DnaK chaperone system-assembled 30S subunits bound tRNA approximately 70% as well as Δ 30S subunits (Table 1). A very low level of tRNA binding was observed for purified RI and Δ RI* particles (Table 1). This low level of binding is likely nonspecific as it is similar to that observed in template-independent reactions (data not shown). The observed difference in the tRNA binding capacity of Δ 30S subunits and chaperone formed 30S subunits is likely not reflective of any significant functional discrepancy, but perhaps purity or stability of the different particles. An additional functional test, subunit association, further supports the authenticity of the chaperone-assembled 30S subunits by demonstrating their competence in forming 70S ribosomes with natural 50S subunits (data not shown). Thus, DnaK, DnaJ, GrpE and ATP, the four components of the DnaK chaperone system, appear to facilitate assembly of functional 30S subunits.

DnaK interacts specifically with a subset of small subunit ribosomal proteins.

To begin to unravel the process by which the DnaK/hsp70 chaperone system acts in 30S subunit assembly the interaction between DnaK and 30S subunit components was investigated. To this end, a complete mixture of individually purified recombinant small subunit ribosomal proteins was assayed for their ability to bind a DnaK affinity column. During the initial washes to remove non-/weakly interacting proteins, some of the DnaK appears to be liberated from the column matrix (Fig. 4A lanes 4-6). However, a significant amount of DnaK can be recovered from the column matrix after a complete round of affinity chromatography (including washes with up to 1 M KCl; data not shown) suggesting that the initial loss is not significant. The identity of the DnaK binding proteins was determined by co-migration with purified ribosomal proteins on SDS-PAGE (Fig. 4A) in combination with

mass spectrometry analysis (data not shown). These data are summarized in Figure 4B.

There is an obvious hierarchy of binding with some of the small subunit proteins appearing to not interact with DnaK, while others are capable of strongly interacting (Fig. 4B). This hierarchical binding is clearly illustrated in the elution patterns of S3 and S4 (Fig. 4A). S3 begins to elute early in the gradient (Fig. 4A lanes 9 and 10), while S4 does not elute from the column until later in the gradient (Fig. 4A lanes 12 and 13) and S4 appears to be one of the last proteins to elute from the DnaK column under these experimental conditions. These interactions could play a role in *in vitro* 30S subunit assembly as a concentration of 330 mM KCl is optimal for this process (Traub and Nomura, 1968; Mizushima and Nomura, 1970; Held et al., 1973; Held et al., 1974).

Interestingly, in addition to the clear divisions based on strength of interaction, the DnaK binding proteins can be divided into three classes based on known properties of the ribosomal proteins. The first class is comprised of those proteins that are stably associated with RI (S4, S8, S16, and S17). Of these, S4, S8 and S17 are primary binding proteins (see Fig. 1A) and thus play a significant role in assembly. The second class is found substoichiometrically associated with RI (S5, S12, and S19) but is found fully associated with Δ RI* (Held et al., 1974), suggesting that the binding of these proteins is altered during the RI to RI* transition. Lastly, the third class binds only after RI* is formed, i.e., is composed of tertiary binding proteins (S3 and S21). The interaction of S3 and S21 with DnaK is striking, as both proteins have been implicated in small subunit function, such as tRNA binding (Ramakrishnan et al., 1986; Vladimirov et al., 1985; Graifer et al., 1989). These results suggest that DnaK may play a role both pre- and post-activation of the 30S subunit assembly intermediate. Thus, the DnaK chaperone system may act to direct formation of functional sites within the 30S subunit, and perhaps play a role in the timing of functional 30S subunit production.

Since DnaK likely binds a variety of proteins while performing its role as a chaperone *in vivo*, the specificity of the interactions between the small subunit proteins and DnaK was assessed by using *E. coli* whole cell lysate in the DnaK affinity chromatography (data not shown). A subset of proteins from the cellular lysate can stably interact with DnaK. Interestingly, the same small subunit ribosomal proteins that are able to bind the DnaK column from a purified system are also found in the subset of cellular proteins associated with DnaK. Thus, these small subunit ribosomal proteins are able to interact with DnaK in the context of the whole cell protein milieu, and likely represent *in vivo* targets for DnaK.

DnaK can be linked to ribosome biogenesis *in vivo*.

Given our data functionally linking DnaK and small subunit components *in vitro*, we investigated the possibility of an *in vivo* interaction. Previously, it had been shown that ribosome biogenesis was altered in a particular allele of dnaK (*dnaK756*; Georgopoulos et al., 1973; Yochem et al., 1978) under non-permissive conditions (high temperature and low salt; Alix and Guerin, 1993). To address whether the interactions we observed between DnaK and the small subunit ribosomal proteins *in vitro* could occur *in vivo*, we assayed for suppression of the temperature-sensitive phenotype of *dnaK756* by overexpression of some of the small subunit ribosomal proteins. Three primary binding proteins (see Fig. 1A), S4, S8 and S15, were chosen as our test set. Each of these proteins plays a critical role in 30S subunit assembly, and moreover each differentially interacts with DnaK as demonstrated in our affinity chromatography experiments (see Fig. 4). No significant interaction between S15 and DnaK was observed, S8 demonstrated an intermediate level of binding and S4 appeared to be one of the strongest DnaK binders. To test for suppression, we cloned the genes for these proteins into a vector that allows inducible overexpression in the *dnaK756* background. We assayed suppression of the temperature-sensitive (ts) growth phenotype by dilution plating (Fig. 5A) and then confirmed trends by measuring doubling times (Fig. 5B).

Overexpression of S4 clearly rescues the ts phenotype of *dnaK756*, although the growth rate has not returned to that observed under permissive conditions (Fig. 5). Nevertheless, the increase in growth in the presence of S4 at high temperature is very striking, particularly since the strain containing S4 grows less well than the strain harboring the empty vector at permissive temperature (Fig. 5A). Overexpression of S15 appears to have no effect on the growth of *dnaK756* (Fig. 5A) while overexpression of S8 affords a slight rescue of *dnaK756* at high temperature (Fig. 5A). These results are intriguing since it appears there is a correlation between the interactions observed by affinity chromatography and the suppression of the ts *dnaK756* phenotype.

Next, we determined if overexpression of S4 could alter the effect of *dnaK756* on ribosome biogenesis. Due to the very slow growth of *dnaK756* at high temperature in low salt media, we initially grew the cultures in rich media (under these conditions *dnaK756* is not temperature sensitive, although for consistency we will refer to the two temperatures as permissive and non-permissive) to allow analysis of polysomes from an equivalent number of cells. At the permissive temperature, polysome profiles from both strains look similar and like those from wild-type strains (Fig. 5Ba and b). However, at the non-permissive temperature the polysome profiles are dramatically different (Fig. 5Bc and d). When *dnaK756* containing the empty vector is grown at high temperature, there is a significant decrease in the number of 70S ribosomes and polysomes are not detected (Fig. 5Bc). In contrast, when *dnaK756* harboring the vector containing S4 is grown at the non-permissive temperature, the polysome profile more closely resembles those from the permissive temperature (Fig. 5B compare d with a and b). The rescue does not appear to be complete, again correlating with the doubling time of the strains (Fig. 5B). Since these strains were grown under conditions where *dnaK756* is not temperature sensitive, it is possible that the full effect of this mutation is not manifested. In an attempt to address this possibility, the

strains were grown under conditions where the temperature sensitive phenotype was apparent. Although far fewer cells could be harvested from the *dnaK756*/vector strain at the non-permissive temperature, analysis of ribosomes revealed a marked change in the distribution of subunits to 70S ribosomes (Fig. 5Be). Thus, it appears that not only the number of ribosomes is altered in this strain but the relative distribution of subunits to ribosomes is also altered. Coordinated synthesis of components for both subunits may account for the observed decrease in both small and large subunits. Characterization of the particles produced under these conditions has proven refractory due to the small amount of material that can be obtained. Overexpression of S4 resulted in more wild-type-like profiles, although again complete rescue was not observed (Fig. 5Bf). These results suggest that DnaK can functionally interact with ribosomal components *in vivo* and that this interaction has an effect on ribosome assembly.

Discussion

Here we have demonstrated that the DnaK/hsp70 chaperone system can facilitate 30S subunit assembly. We have shown that components of the DnaK chaperone system (DnaK and GrpE) can stably bind pre-30S particles (Fig. 2). Hydrolysis of ATP in combination with the action of DnaK, DnaJ and GrpE are all involved in facilitating 30S subunit assembly (Fig. 3). We have also presented evidence suggesting *in vitro* and *in vivo* interactions between DnaK and 30S subunit components (Figs. 4 and 5). Ribosome biogenesis is also altered by these *in vivo* interactions (Fig. 5). These results have led us to propose a model for 30S subunit assembly (Fig. 6). This model reflects data presented here and the likelihood that the chaperone components function in 30S subunit assembly in a manner similar to their proposed functions in protein folding (Burston and Clarke, 1995).

Generally, DnaK is assisted in substrate binding by DnaJ (Schmid et al., 1994). Therefore by analogy in our model (Fig. 6), DnaK binds to the substrate RNP, in a reaction

potentially augmented by DnaJ and ATP (or ATP hydrolysis). GrpE, which can be found associated with the intermediate (Fig. 2C), is likely tethered to the complex by its well-documented interaction with DnaK (Schonfeld et al., 1995; Wu et al., 1996). When DnaK is in an ADP-bound state, it likely remains bound to the RNP. It is possible that DnaK binding is responsible for the RI to RI* transition and that continued association of DnaK with the RNP could block further assembly. If the role of GrpE in 30S subunit assembly is similar to that in protein folding, it is likely involved in exchange of ADP/Pi for ATP on DnaK and that this exchange results in conformational changes within DnaK which occur concomitantly with release of RI* from the chaperones. These changes could result in the transition of RI to RI* (if DnaK binding is not sufficient for this transition) and may include an initial interaction with the tertiary binding proteins. Once released from DnaK, the rearranged, competent intermediate could undergo the final stages of assembly to form a functional 30S subunit. Given the complexity of 30S subunit assembly and the reactions facilitated by the DnaK chaperone system, it seems unlikely that the mechanism of DnaK chaperone system-facilitated 30S subunit assembly will prove trivial to dissect.

The interaction of the substrate RNP with DnaK could occur directly via DnaK binding to one or more of the small subunit proteins, or DnaK may have affinity for 16S rRNA as well. An interaction between DnaK and 16S rRNA would not be completely unprecedented; it has been shown that chaperones belonging to both the hsp60 and hsp70 systems can interact with RNA (Ruggero et al., 1998; Okada et al., 2000; Zimmer et al., 2001). Recent work has also shown that the binding of DnaK to RNA is affected by the presence of its co-chaperones GrpE and DnaJ (Zimmer et al., 2001). Therefore, the interaction between the DnaK chaperone system and the 30S subunit assembly intermediate RNP may be mediated by 16S RNA. Alternatively, DnaK may bind directly to some of the small subunit ribosomal proteins. The proteins that were identified as DnaK binding proteins

(Fig. 4) are good candidates for mediating these interactions. In addition, some of the unique structural features of the small subunit ribosomal proteins (Brodersen et al., 2001) could also prove important for DnaK chaperone system binding to the 30S subunit assembly intermediate RNP. These interactions might occur in a manner analogous to those documented for protein substrate binding (see Bukau and Horwich, 1998 and Fink, 1999 for reviews). Hence, RNA or protein binding may mediate the association of DnaK with the pre-30S particles. Moreover, a combination of these binding events may play a role, as they are not mutually exclusive. Also it is possible that features unique to this RNP are involved in binding. Regardless of the specifics, it appears that components of the DnaK chaperone system act in concert in RNA, RNP and protein binding/folding, and that the mechanisms involved in the interaction with/folding of these different substrates may share similar characteristics.

A role for DnaK/hsp70s in ribosome assembly has previously been suggested. Growth of a temperature-sensitive mutant *dnaK* strain (*dnaK756*) at the non-permissive temperature resulted in a defect in ribosome biogenesis (Alix and Guerin, 1993). However, defects in other cellular processes are also observed in this *dnaK* strain, and thus the specificity of the alteration on ribosome assembly has not been easy to address. Here we have demonstrated that overexpression of small subunit proteins that bind DnaK (see Fig. 4) can partially suppress the temperature-sensitive phenotype and partially rescue the ribosome assembly defect of this *dnaK* allele (see Fig. 5). The known roles and structures of these proteins may offer insight into how DnaK influences 30S subunit assembly. A role of hsp70s in ribosome biogenesis may not be limited to *E. coli*. Two hsp70 homologues, Ssa1p and Ssb1p, have been found associated with ribosome assembly intermediates in yeast (Harnpicharnchai et al., 2001). Thus, it appears that the participation of hsp70 chaperone systems in ribosome biogenesis may be a highly conserved phenomenon.

Conclusions

Here we present findings that offer evidence for a role of the DnaK/hsp70 chaperone system in 30S ribosomal subunit assembly *in vitro*. Studies presented here also suggest that DnaK and small subunit components functionally interact *in vivo*. In addition to revealing the possible existence of ribosome assembly factors, these findings link two very highly conserved systems, ribosomes and hsp70s, thus raising the possibility that hsp70 chaperone systems act during ribosome assembly throughout phylogeny. However, given that DnaK is not essential (Paek and Walker, 1987) and the cellular importance of an active pool of ribosomes, the intriguing possibility exists that there are other yet to be identified factors involved in ribosome biogenesis.

Experimental Procedures

In vitro Reconstitution, Gradient Fractionation and Isolation/Purification of Particles

In vitro reconstitution of 30S subunits, sucrose gradient sedimentation, purification and concentration of ribosomal particles and protein analysis were performed essentially as described by Culver and Noller (1999 and 2000). In all reconstitutions, final concentration of 16S rRNA was 0.4 μM , that of each recombinant protein was 2.8 μM , and that of TP30 was 0.6 μM . Reconstitutions were performed at 15°C with incubations at 42°C designated by Δ . Reconstituted particles were concentrated using Centricon 100s (Amicon) except for those shown in Figure 2B where Centricon 3s were used. For all sucrose gradient sedimentation experiments, top and bottom indicate sedimentation direction, and absorbance was monitored at 254 nm.

Reconstitutions using chaperones (and the corresponding controls) contained 100 μM ATP (final concentration) except Figure 3A panel c, where 100 μM ATP γ S was used in place of ATP. Also, reconstitution reactions including chaperones (and the associated control

reconstitutions) contained 10% glycerol (v:v, final concentration), 0.4 μ M DnaK, 0.4 μ M DnaJ, and 0.8 μ M GrpE.

S100 Extract Preparation

S100 extract was prepared by resuspending 10 g of *MRE600 E. coli* in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 22 mM NH_4Cl and 6 mM β -mercaptoethanol (BME). Cells were lysed by passing them through a French Press twice (12,000 psi). Cell lysate was cleared by centrifugation at 16,000 rpm (JA-20) at 4°C. The cell lysate was centrifuged at 33,000 rpm for 12 hours in a Ti-70 at 4°C. Supernatant was loaded onto a 40 mL DE52-cellulose column and washed with 400 mL of the extract buffer. The enzyme fraction was eluted with the same buffer except the NH_4Cl concentration was 250 mM. Fractions were aliquoted and stored at -80°C.

Protein Sequence Analysis

Preparation of the protein sample for N-terminal sequence analysis was performed essentially as described by Matsudaira (1987) and sequencing was performed by the Protein Facility at Iowa State University.

Western Analysis

Anti-DnaK, -GrpE, -DnaJ and secondary antibodies were purchased from StressGen (Victoria, BC). Amersham-Pharmacia ECL western blot analysis kit was used as described in company literature for analysis. Isolated and concentrated particles (see above and Culver and Noller, 2000) were precipitated with 5 volumes of ice-cold acetone and the resulting pellets were analyzed resulting in a non-quantitative loading.

Chaperone components

Recombinant DnaK was produced and purified using an expression system based on the IMPACT system (New England Biolabs) generously provided by Anthony L. Fink and Charles Sargenti, University of California, Santa Cruz. Strain *ER2566* containing DnaK

cloned into pTYB1 was grown to an OD₅₅₀ of 0.6-0.8 in 2xYT at 37°C. Cultures were induced by addition of 1 mM IPTG (final concentration) and grown at 17°C for 6 hours. Cells were harvested by centrifugation at 5,000 rpm for 10 minutes, washed once with Buffer K [20 mM K⁺-Hepes (pH 7.6) and 330 mM KCl] and then stored at -80°C. Cells were disrupted by sonication in 6 mls of Buffer K at 4°C and all subsequent steps were performed at 4°C. The resulting lysate was cleared by centrifugation for 15 minutes at 5,000 rpm. The supernatant was loaded on a 3 mL chitin column (New England Biolabs) that was previously equilibrated in Buffer K. The column was washed with 24 mL of Buffer K. DTT was added (final concentration of 40 mM) and the column was sealed and gently rocked overnight. The matrix was allowed to settle prior to elution of cleaved DnaK with 18 mL of Buffer K. Fractions containing DnaK were identified by SDS-PAGE analysis (Laemmli, 1970) and then stored at either 4°C or -80°C. Protein concentration was determined by Bradford assay. For reconstitutions, purified DnaK, GrpE, and DnaJ were purchased from StressGen.

Transfer RNA binding

Transfer RNA binding was performed as described in Culver and Noller, 1999.

DnaK Affinity Chromatography

Affinity chromatography was performed following the general procedure of Kellogg and Alberts (1992). A DnaK affinity column matrix (1mL) was prepared using a DnaK-intein-chitin fusion protein (see above). The DnaK fusion occurs at the very C-terminus of DnaK and is likely well enough removed from the active site to not interfere with substrate binding. An extract containing the DnaK-intein-chitin fusion protein (prepared as described above) was applied to a chitin binding domain matrix. Cellular proteins (which should not bind the matrix) were eluted by washing with 7 column volumes of Buffer K followed by 3 column volumes of Buffer K(low) [20 mM K⁺-Hepes (pH 7.6) and 50 mM KCl]. A mixture

of a complete set of purified recombinant small subunit ribosomal proteins (6000 pmol total) was applied to the column matrix in Buffer K(low) and allowed to bind to the matrix as a batch overnight. The matrix was allowed to settle and unbound proteins were removed in flow through and subsequent wash steps with Buffer K(low). Bound proteins were eluted in a series of steps with increasing KCl concentration from 100 mM to 1 M KCl. A final step cleaved DnaK from the column using 40 mM DTT to ensure that DnaK remained stably bound to the matrix throughout the procedure. SDS-PAGE analysis (see above) of the individual column fractions was performed after concentration by precipitation with 5 volumes of ice-cold acetone.

Bacterial Strains, Plasmids and Growth Conditions

E. coli strain *dnaK756* (Georgopoulos et al., 1973; Yochem et al., 1978; originally *groPC756*) was obtained from the *E. coli* Genetic Stock Center. For expression in the *dnaK756* background, the genes encoding S4, S8 and S15 were subcloned from previously prepared plasmids that contain the wild-type genes (Culver and Noller, 1999). Fragments (NdeI and EcoRI) containing the wild-type genes were ligated into the pALTER-1 vector (Promega) digested with the same enzymes. Ligation reactions were transformed into XL-1Blue Kan^r cells (Stratagene) and clones were confirmed to have the appropriate inserts. The pALTER vector, pALTER/S4, pALTER/S8 and pALTER/S15 DNAs were then transformed into *dnaK756*.

For the dilution plating, equal number of cells from overnight cultures grown in Luria Broth (LB) + 10 µg/ml tetracycline (tet) at 37°C were sub-cultured into low salt broth (LSB; same as LB except 1/10 as much sodium chloride) + 10 µg/ml tet + 1 mM IPTG (final concentration) and grown at either 37°C (permissive) or 43°C (non-permissive) for 3.5 hours. Ten-fold serial dilutions were made using LSB and were plated on LSB plates containing 10 µg/ml tet. Plates were incubated at 37°C for 14 hours or at 42°C for 60 hours. For doubling

time calculations, cells were grown in liquid as described above except OD₆₀₀ measurements were made at various times. Cultures that were grown at 42°C were induced with 1 mM IPTG (final concentration) at time zero while cultures that were grown at 37°C were induced after 70 minutes.

Polysome Analysis

Polysomes were prepared based on standard protocols with some slight modifications. Briefly, equal number of cells from overnight cultures grown in LB + 10 µg/ml tet at 37°C were sub-cultured into LB or LSB containing 10 µg/ml tet and 1 mM IPTG (final concentration) and grown at either 37°C (permissive) or 42°C (non-permissive) for 8.5 hours. OD₅₅₀ measurements were taken at this time. Cell cultures were rapidly chilled by pouring over ice. Cells were harvested by centrifugation at 10,000 rpm for 5 minutes at 4°C. Cell pellets were resuspended in 0.5 ml chilled lysis buffer (10 mM Tris-HCl (pH 7.8), 15 mM MgCl₂, 1 mg/ml lysozyme) and frozen in a dry ice/ethanol bath. Cells were thawed in an ice water bath, refrozen in a dry ice/ethanol bath, and then stored at -80°C until needed. To finish cell lysis, cells were thawed in an ice water bath. 15 µl 10% deoxycholate was added to each sample and the samples were centrifuged at 13,200 rpm for 20 minutes at 4°C. For polysomes that were prepared from cultures grown in LB, lysate from an equal number of cells was loaded on onto a 10-40% sucrose gradient containing 20 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 100 mM NH₄Cl, and 2 mM DTT. Gradients were centrifuged in an SW-41 rotor for 3.5 hours at 35,000 rpm at 4°C. For polysomes prepared from cultures grown in LSB, a complete correction for different number of cells could not be made due to the lack of growth at 42°C for some strains; therefore panels E and F of Figure 5 should not be over-interpreted.

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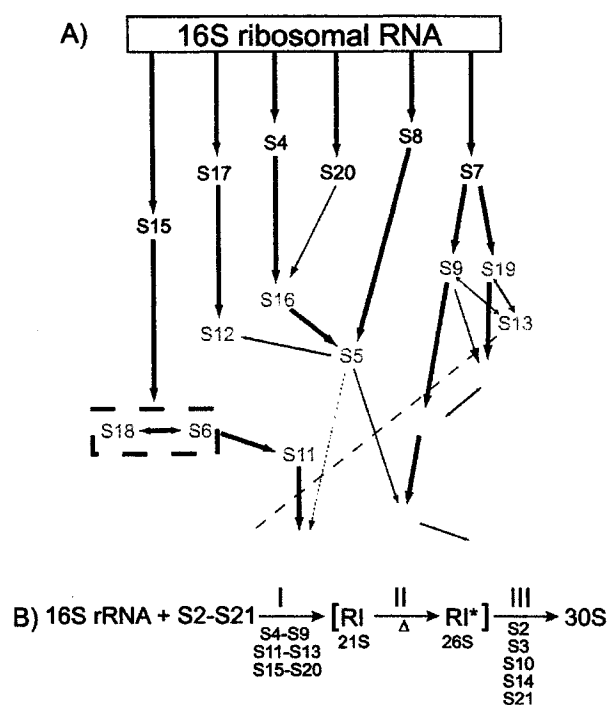


Figure 1. *In vitro* Assembly of *E. coli* 30S Ribosomal Subunits.

A. Simplified *in vitro* 30S subunit assembly map (Mizushima and Nomura, 1970; Held et al., 1974; G.M.C. in press). 16S rRNA is represented as a rectangle. Arrows indicate interactions between components. Primary (1°) binding proteins are black, secondary (2°) binding proteins are purple and tertiary (3°) binding proteins are blue. S6 and S18 are shown in a dashed line box to indicate that they bind as a heterodimer. The dotted line indicates the separation of proteins found on the Reconstitution Intermediate (RI: above the line) from those that are added after activation (below the line).

B. Schematic path for *in vitro* 30S subunit assembly. RI, Reconstitution Intermediate. RI*, Activated Reconstitution Intermediate. I designates incubation at low temperature. Δ and II designate heat activation at 42°C. III designates incubation at either low or high temperature.

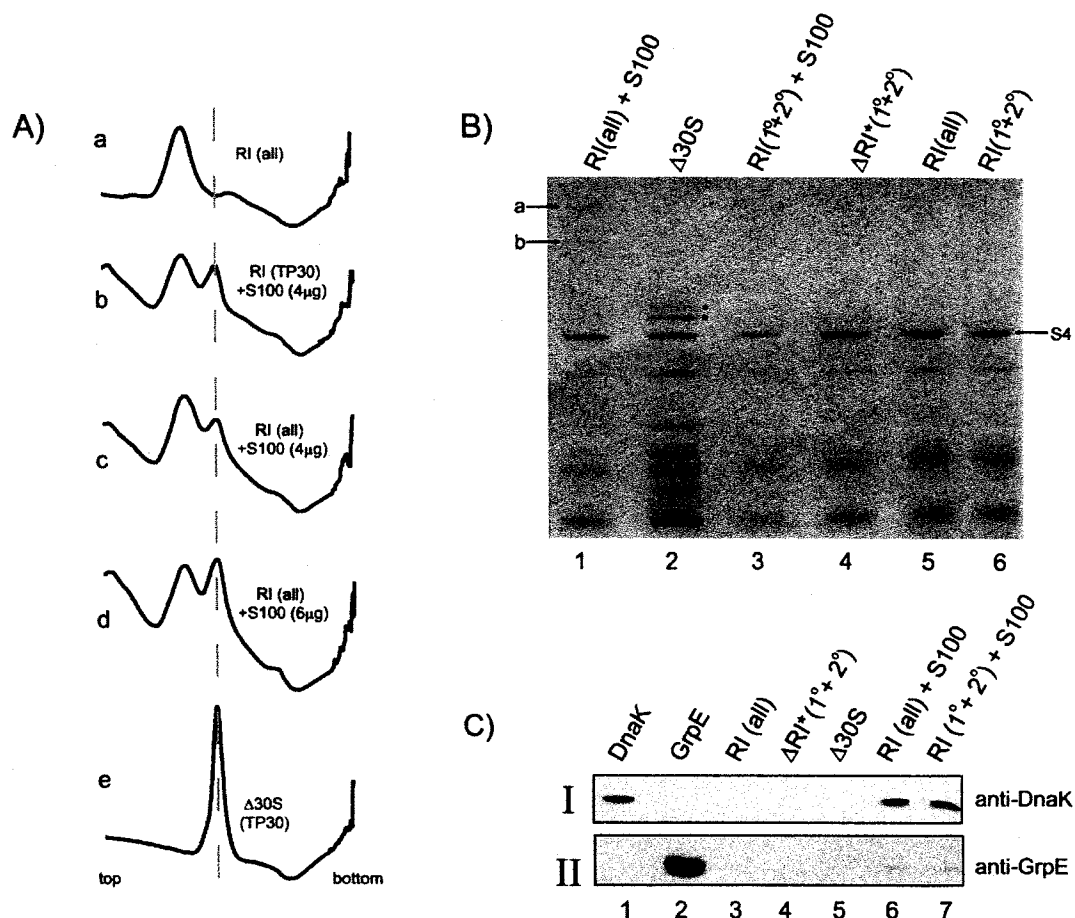


Figure 2. Extract Facilitated *in vitro* 30S Subunit Assembly.

Reconstitutions are incubated at 15°C for the complete reaction unless otherwise noted.

Proteins used in the reconstitutions are noted below.

A. Sucrose gradient sedimentation analysis of *in vitro* reconstituted 30S subunits treated with partially purified *E. coli* extract. Dotted line indicates the position of 30S subunits. a) RI (all),

Reconstitutions performed using a complete set of recombinant small subunit ribosomal proteins. b) RI (TP30) + S100 (4 μ g), Reconstitutions performed using TP30 with 4 μ g of S100 extract added after RI formation. c) RI (all) + S100 (4 μ g), Reconstitutions performed using a complete set of recombinant small subunit ribosomal proteins with 4 μ g of S100 extract added after RI formation. d) RI (all) + S100 (6 μ g). Reconstitutions performed using a complete set of recombinant small subunit ribosomal proteins with 6 μ g of S100 extract added after RI formation. e) Δ 30S (TP30), Reconstitutions performed using TP30 with a shift to 42°C after RI formation at 15°C.

B. SDS-PAGE analysis of proteins derived from sucrose gradient purified reconstituted particles. Proteins indicated by a and b are non-ribosomal proteins. Positions of S2 and S3 are indicated by dots and the position of S4 is indicated by an arrow. 1) RI (all) + S100, Particles formed using a complete set of recombinant small subunit ribosomal proteins incubated with 4 μ g of S100 extract added after RI formation. 2) Δ 30S, Particles formed using a complete set of recombinant small subunit ribosomal proteins with a shift to 42°C after RI formation. 3) RI (1° + 2°) + S100, Particles formed using the recombinant primary (1°) and secondary (2°) small subunit ribosomal proteins with 4 μ g of S100 extract added after RI formation. 4) Δ RI* (1° + 2°), Particles formed using the recombinant primary (1°) and secondary (2°) small subunit ribosomal proteins with a shift to 42°C after RI formation. 5) RI(all), Particles formed using a complete set of recombinant small subunit ribosomal proteins. 6) RI (1° + 2°), Particles formed using the recombinant primary (1°) and secondary (2°) small subunit ribosomal proteins.

C. Western analysis of sucrose gradient purified reconstituted particles. Particles were formed at 15°C unless otherwise noted. Panels I and II were probed using monoclonal anti-DnaK and anti-GrpE antibody respectively. The same approach and results were observed using extract treated particles or particles formed with purified chaperone components. The membrane was first probed with anti-GrpE then stripped and probed with anti-DnaK. 1) DnaK, 1 µg purified DnaK. 2) GrpE, 1 µg purified GrpE. 3) RI (all), Particles formed using a complete set of recombinant small subunit ribosomal proteins. 4) Δ RI*(1°+2°), Particles formed using the recombinant primary (1°) and secondary (2°) small subunit ribosomal proteins with a shift to 42°C after RI formation. 5) Δ 30S, Particles formed using a complete set of recombinant small subunit ribosomal proteins with a shift to 42°C after RI formation. 6) RI (all) +S100, Particles formed using a complete set of recombinant small subunit ribosomal proteins with 4 µg of S100 extract added after RI formation. 7) RI (1°+2°) +S100, Particles formed using the recombinant primary (1°) and secondary (2°) small subunit ribosomal proteins with 4 µg of S100 extract added after RI formation.

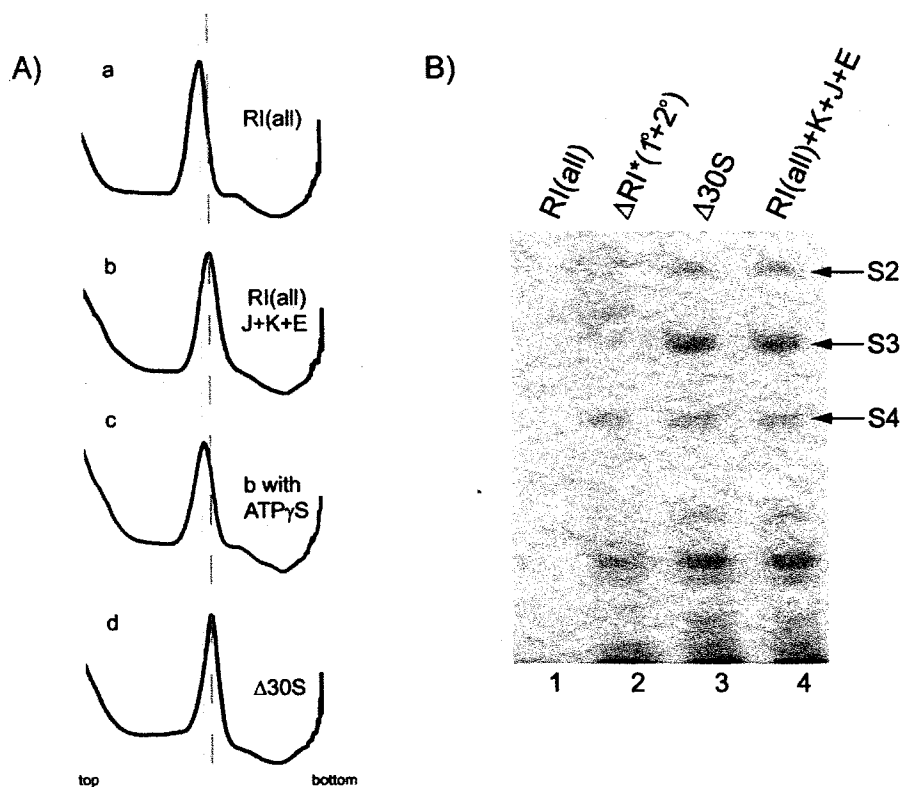


Figure 3. Purified DnaK chaperone system components facilitate 30S subunit assembly at low temperature.

All reconstitutions contained the complete set of recombinant small subunit proteins, 100 μ M ATP and were performed at 15°C unless otherwise noted.

A. Sucrose gradient sedimentation analysis of *in vitro* 30S subunit reconstitutions using purified DnaK chaperone system components. Dotted lines indicate the position of RI (lighter) and 30S subunits (darker). a) RI (all), Reconstitution using a complete set of recombinant proteins. b) RI (all) J+K+E, Reconstitution as described in a with DnaK, DnaJ, and GrpE. c) b with ATP γ S, Reconstitution as described for b with ATP γ S in place of ATP. d) Δ 30S, Reconstitution as described in a were shifted to 42°C after the initial incubation.

B. SDS-PAGE analysis of sucrose gradient purified *in vitro* formed particles using purified DnaK chaperone system components. The positions of the primary binding protein S4 and the tertiary binding proteins S2 and S3 are indicated. 1) RI (all), Reconstitution using a complete set of recombinant proteins. 2) Δ RI*, Reconstitution using only the primary (1^o) and secondary (2^o) small subunit ribosomal proteins with a shift to 42°C after RI formation. 3) Δ 30S, Reconstitution as described for 1 was shifted to 42°C after RI formation. 4) RI (all) J+K+E, Reconstitution using a complete set of recombinant proteins with DnaK, DnaJ, and GrpE.

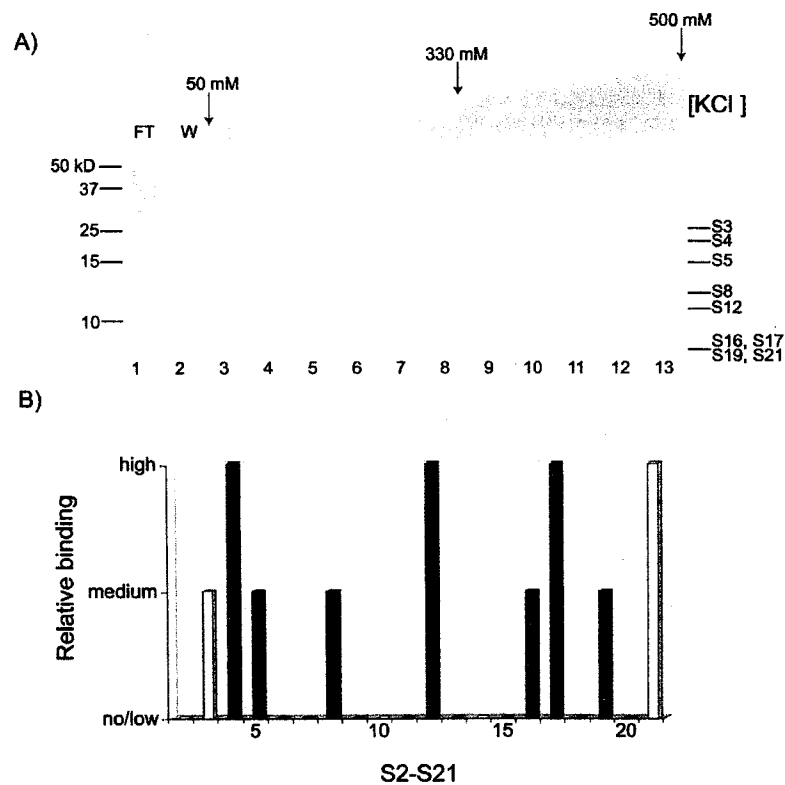


Figure 4. A subset of small subunit ribosomal proteins binds to DnaK.

A. SDS-PAGE analysis of the DnaK affinity chromatography with purified small subunit ribosomal proteins. 1) FT, flow through of extract in preparation of the DnaK affinity column. 2) W, first wash after applying DnaK-intein-chitin protein containing extract. 3) flow through after application and overnight incubation of the purified small subunit ribosomal proteins with the DnaK column. 4-7) successive washes with buffer containing 50 mM KCl. 8) proteins eluted at 100 mM KCl. 9-13) proteins eluted with increasing concentrations of KCl from 330 to 500 mM.

B. Relative elution patterns of small subunit ribosomal proteins during DnaK affinity chromatography. Coloring reflects that shown in Figure 1A. No/low, no detectable binding at 50 mM KCl; medium, initial elution observed upon addition of 330 mM KCl; high, initial elution observed upon addition of 500 mM KCl.

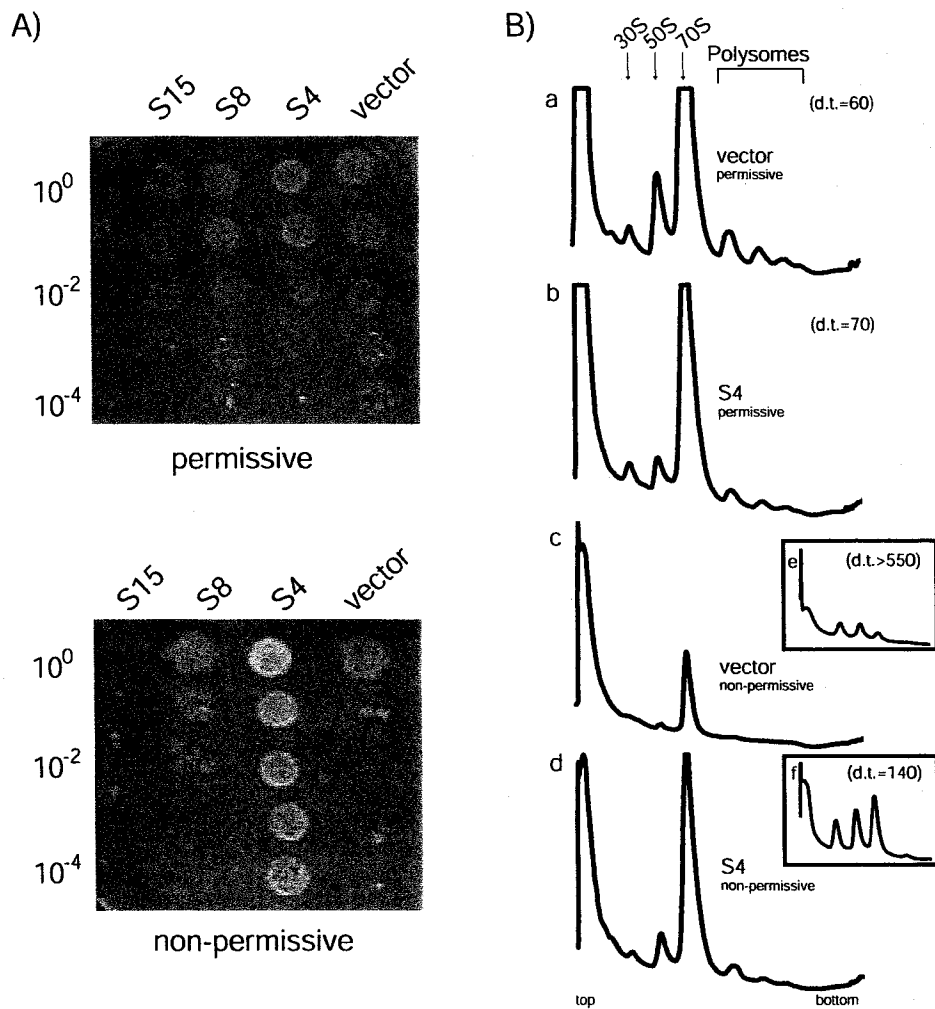
dnaK756 +

Figure 5. DnaK can be linked to Ribosome Biogenesis *in vivo*.

A) Growth of the *dnaK756* mutant under various conditions. 10-fold serial dilution of *dnaK756* containing different constructs. permissive, 37°C. non-permissive, 43°C. vector, *dnaK756* harboring the empty pALTER vector. S4, *dnaK756* harboring the pALTER vector

containing the gene for S4. S8, *dnaK756* harboring the pALTER vector containing the gene for S8. S15, *dnaK756* harboring the pALTER vector containing the gene for S15.

B. Sucrose gradient sedimentation analysis of polysomes isolated from *dnaK756* mutant grown under various conditions. Positions of 30S and 50S subunits, 70S ribosomes, and polysomes are indicated. Doubling times (d.t.) are given in minutes and shown in parentheses. Polysomes shown in panels a-d were isolated from cells grown in LB.

Polysomes shown in the inset panels e and f were isolated from cells grown in LSB. a) vector, permissive; *dnaK756* harboring the empty pALTER vector grown at 37°C. b) S4, permissive; *dnaK756* harboring the pALTER vector containing the gene for S4 grown at 37°C. c and e) vector, non-permissive; *dnaK756* harboring the empty pALTER vector grown at 42°C. d and f) S4, non-permissive; *dnaK756* harboring the pALTER vector containing the gene for S4 grown at 42°C.

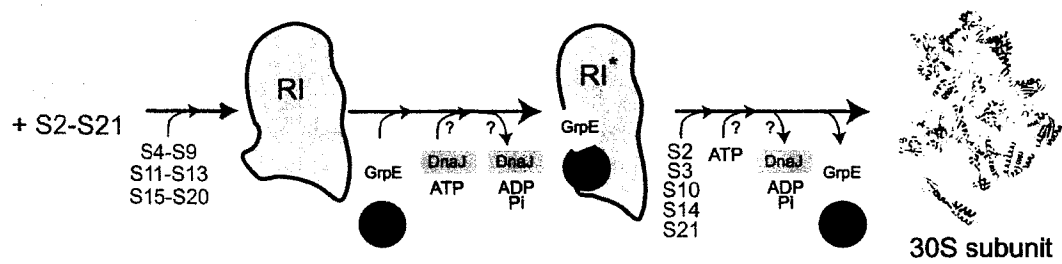


Figure 6. Putative model for 30S subunit assembly.

RI, Reconstitution Intermediate RI*, Activated Reconstitution Intermediate. The 30S subunit structure is that of the *T. thermophilus* 30S subunit (Wimberly et al., 2000) and this portion of the figure was prepared using Ribbons (Carson, 1997).

Table 1.**Transfer RNA Binding of Reconstituted 30S Particles**

Particle	% Binding
Δ30S	100 \pm 4
RI (all) + K/J/E	68 \pm 7
RI (all)	4 \pm 5
ΔRI*	3 \pm 2

Filter binding of tRNA was performed as previously described for recombinant reconstituted ribosomal particles (Culver and Noller, 1999, 2000).

**CHAPTER 3. DEMONSTRATION OF THE ROLE OF
THE DNAK CHAPERONE SYSTEM IN ASSEMBLY OF 30S
RIBOSOMAL SUBUNITS USING A PURIFIED *IN VITRO* SYSTEM**

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Abstract

Recently, there has been controversy regarding the ability of the DnaK chaperone system to facilitate *E. coli* 30S subunit assembly at otherwise non-permissive conditions. Here we present additional data indicating that purified DnaK chaperone assembled 30S subunits are functional. Additionally, explanations for the reported differences are discussed.

Introduction

Ribosome assembly is a highly complicated process that, despite years of work and the continuing efforts of many groups, is still not very well understood [see (Williamson, 2003) for overview]. One avenue of research has been directed toward identifying extrinsic factors that facilitate assembly [see ((Alix and Guerin, 1993; Charollais et al., 2003) for examples]. Recently, Alix and Nierhaus (2003) have reported experiments that appear to contradict our previous results showing that the DnaK chaperone system facilitates 30S subunit assembly under otherwise non-permissive conditions (Maki et al., 2002). Here, we

take the opportunity to reexamine the experiments performed by Alix and Nierhaus and to offer alternative explanations for the reported differences.

To briefly summarize our findings (Maki et al., 2002), we first searched for cellular factors that facilitate 30S subunit assembly *in vitro*. Toward this end, we focused on a well-known stall in assembly that occurs at low temperature and results in the production of an assembly intermediate, the 21S, RI (Reconstitution Intermediate) particle (Held and Nomura, 1973; Traub and Nomura, 1968a). RI contains a subset of the small subunit ribosomal proteins and requires heat activation to assemble a full complement of proteins into a functional 30S particle. We observed that treatment of the intermediate with S100 extract at the non-permissive temperature resulted in the formation of a peak that co-sediments with 30S subunits, suggesting that factors which facilitate assembly were present in the extract. A non-ribosomal protein was found associated with the intermediate, and N-terminal sequencing in combination with western blot analysis identified this protein as DnaK. The purified DnaK chaperone system, (DnaK, its two co-chaperones, DnaJ and GrpE, and ATP) was shown to be sufficient to convert the sedimentation of the 21S, RI particle to something resembling a 30S peak at the non-permissive temperature. The appropriate ribosomal proteins were associated with this 30S peak after sucrose gradient sedimentation and purification. The purified particle was shown to have tRNA binding activity significantly above that of the assembly intermediate, though not as robust as the tRNA binding activity of 30S subunits formed by heat activation. Additionally, we demonstrated that DnaK selectively interacts with a subset of small subunit ribosomal proteins, of which S4 showed the strongest binding. Lastly, it was observed that overexpression of S4 could partially rescue phenotypic defects observed for a temperature sensitive allele of *dnaK* (*dnaK756*). From these results, we concluded that the DnaK chaperone system facilitates 30S subunit assembly.

In their paper “DnaK-facilitated ribosome assembly in *Escherichia coli* revisited”, Alix and Nierhaus (2003) used two main approaches to test our conclusions. In the first, they assessed the kinetics of *in vitro* 30S subunit reconstitution at various temperatures in the presence and absence of the DnaK chaperone system by monitoring poly(U)-directed polyphenylalanine synthesis. The same levels of polyphenylalanine synthesis in the presence and absence of purified chaperone components were observed at all tested temperatures. In the second approach, ribosomal components from an *E. coli dnaK* null strain (BB1553; grown at the permissive temperature) were compared to wild-type components. Ribosomal subunits, both 30S and 50S, reconstituted with components isolated from the *dnaK* knockout strain were shown to participate in polyphenylalanine synthesis assay. Also, ribosomal proteins isolated from the *dnaK* null strain grown under permissive conditions were shown to be similar to proteins isolated from a wild-type strain. Lastly, ribosomal subunits from both the *dnaK* knockout and wild-type strains were stable when incubated up to 50° C in a 1 mM MgCl₂, 200 mM NH₄Cl buffer. From these studies, Alix and Nierhaus (2003) conclude, “the DnaK chaperone family is not sufficient to facilitate reconstitution of 30S subunits...”

There are two main differences in the functional experiments performed by Alix and Nierhaus (Alix and Nierhaus, 2003) and those performed by us (Maki et al., 2002) that warrant discussion. The first involves the manner in which the chaperone treated reconstituted 30S subunits are handled prior to being assayed for function. Alix and Nierhaus (2003) assayed crude 30S subunit reconstitution mixtures for polyphenylalanine synthesis capability. In marked contrast, we used reconstituted 30S particles which were sucrose gradient purified and then washed and concentrated on molecular sieving filters (molecular weight cut-off of 100,000; (Maki et al., 2002). It appears that assaying purified particles is important for monitoring function of different populations of reconstituted 30S subunits, as we too have difficulties detecting activity of crude reconstitution mixtures

prepared in the presence of the DnaK chaperone system at low temperature. In the absence of purification, it is possible that DnaK remains bound to the 30S subunit, inhibiting function. Indeed, western blot analysis reveals that DnaK is still bound to particles that were reconstituted at low temperature in the presence of the DnaK chaperone system and then isolated from a sucrose gradient (Figure 1, lane 2). Conversely, when the same sucrose gradient purified particles are concentrated on Centricon 100s and washed with reconstitution buffer (containing 330 mM KCl) prior to western blot analysis, significantly less DnaK is found associated with the particles (Figure 1, lane 3). To allow for semi-quantitative results, equal amounts of particles were examined and serial dilution of samples was also performed. Additionally, the same results were obtained using various reconstitution conditions; however at higher reconstitution temperatures the amount of DnaK that remains bound appears to be somewhat diminished. These results suggest that the observed differences in functional capability of reconstituted 30S subunits could be due to the presence of DnaK. Our use of purified 30S particles is also relevant to the question raised by Alix and Nierhaus of possible assembly during our tRNA binding assay. Given our purification procedure, only proteins that were stably associated with the 16S rRNA-containing particle were present in the tRNA binding assay. This precludes the binding of additional non-associated proteins during the tRNA binding assay at 37°C. Thus, incubation at 37°C alone could not account for our results. Our previous results in conjunction with those presented here suggest that removal of DnaK from 30S subunits during their assembly is an important step in functional 30S subunit formation.

The second difference between our experiments (Maki et al., 2002) and those of Alix and Nierhaus (2003) involves the chosen functional assay. In our previous studies, we used tRNA binding to monitor the function of reconstituted 30S subunits. We chose this assay because tRNA binding is an inherent function of the 30S subunit and thus is highly sensitive

to defects in its assembly and structure. In contrast, Alix and Nierhaus (2003) monitored polyphenylalanine synthesis that is dependent on the presence of natural 50S subunits, which can often mask deficiencies in 30S subunit function. The polyphenylalanine synthesis experiments performed by Alix and Nierhaus (2003) involved the addition of an *E. coli* crude high-speed supernatant fraction, S150 (Alix and Nierhaus, 2003). We previously demonstrated that all of the DnaK chaperone components are present in an S100 extract (Maki et al., 2002) and subsequently have prepared S150 extract as described by Alix and Nierhaus (2003; (Nierhaus, 1990). Western blot analysis of S150 extract reveals that DnaK, DnaJ and GrpE are all present at levels comparable to those found in S100 extract (Figure 2). Thus, while the authors went to some lengths to determine that the 30S subunits used to prepare the components for these experiments were devoid of DnaK, they appear to have overlooked another source of chaperone contamination. Hence, the question of whether the presence of the DnaK chaperone system alters the kinetics of 30S subunit assembly at various temperatures remains unanswered.

We have now monitored function of sucrose gradient purified and washed reconstituted 30S subunits, using a completely defined polyphenylalanine synthesis assay. This system utilizes purified translation factors and pre-charged tRNAs (Southworth et al., 2002), eliminating the requirement for S100 or S150 extract and thus circumventing the chaperone contamination problem. These experiments demonstrate that 30S subunits assembled at low temperature in the presence of the DnaK chaperone system are more active than the 21S intermediate, but less active than 30S subunits formed by heat activation (Figure 3). While the percent activity of the chaperone treated 30S particle relative to heat activated 30S subunits is similar to what we reported for tRNA binding, the activity of RI is greatly increased relative to our reported results. For these experiments, we performed the reconstitutions at 20°C, following the protocol of Alix and Nierhaus (2003), which might

partially account for this increased activity; in our hands, at 20°C, a sub-population of 21S particles are able to form 30S particles in the absence of any additional factors. These changes in the reconstitution conditions and in the functional assay performed, which is dependent on the activity of natural 50S subunits, result in an apparent lower level of stimulation by the DnaK chaperone system. However, in these experiments what is actually decreased is the dynamic range of these measurements. Nonetheless, the results of our polyphenylalanine synthesis assays (Figure 3) are consistent with our previous conclusions that the DnaK chaperone system facilitates assembly of functional 30S subunits under otherwise non-permissive conditions.

A final point of discussion is whether DnaK plays a role in ribosome biogenesis *in vivo*. Toward this end Alix and Nierhaus (2003) examined ribosomes and ribosomal components formed in a *dnaK* null strain. They observed that there is virtually no difference in thermostability between ribosomes isolated from *dnaK* knockout or wild-type strains, when the knockout is grown *under the permissive conditions* (Alix and Nierhaus, 2003). This is not surprising, as Alix and colleagues have previously reported that “...DnaK acts during ribosome assembly itself, and not by stabilizing mature ribosomes or protecting them from thermal injury (Alix and Guerin, 1993)” (Hage et al., 2001). Thus, while the thermostability of ribosomes isolated under permissive conditions from the $\Delta dnaK$ strain might be of interest, its bearing on our findings is questionable. Addressing our *in vivo* results, Alix and Nierhaus (2003) suggested that the partial suppression by S4 (at the non-permissive temperature) of the altered polysome profiles in *dnaK756* (a temperature sensitive allele of *dnaK*) is due to its role as a translational repressor. This is a possibility; however, overexpression of S8, another translational repressor, did not yield the level of suppression observed with S4 (Maki et al., 2002). This suggests that changes in control of ribosome component production could not solely account for these results. Therefore, while the role of

the DnaK chaperone system in ribosome biogenesis *in vivo* is still not well understood, it remains clear that a role for these chaperones in 30S subunit assembly can be demonstrated using a purified *in vitro* system.

The assembly of ribosomes is a highly complicated process. Given the importance of ribosome assembly to cell viability there are likely many factors that are involved in this process. To date our approach of searching for extrinsic 30S subunit assembly factors has focused on a small step in this assembly process and thus far led us to the DnaK chaperone system. As previously suggested (Maki et al., 2002), since ribosome biogenesis is of paramount importance and since *dnaK* is not essential (Paek and Walker, 1987), it is likely that other factors involved in ribosome biogenesis have yet to be identified. This is consistent with conclusions of Alix and colleagues (Hage et al., 2001): “DnaK is necessary [for ribosome biogenesis] above 42°C, but not below that temperature, *provided* that other HSPs are constitutively expressed...” Therefore, it follows that additional factors also facilitate ribosome assembly. We agree with Alix and Nierhaus that more sophisticated approaches for studying assembly are warranted and hope that advances can be made in this area. However, until such time, continued efforts using available approaches and minimal, purified systems will hopefully allow identification of an ensemble of ribosome biogenesis factors.

Acknowledgements

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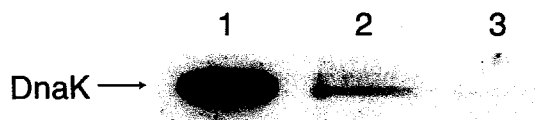


Figure 1. Western Blot Analysis of *in vitro* reconstituted 30S particles.

Reconstituted 30S subunits were formed at 15°C under the following conditions: 16S rRNA:DnaK:DnaJ:GrpE 1:1:1:2. The resulting particles were applied to 10-40% sucrose gradients (see Culver and Noller, 1999), the peak was collected and split in two equal fractions. One fraction was directly precipitated, while the second was concentrated and washed (with a buffer containing 330 mM KCl) on a Centricon 100 sieving filter prior to precipitation. Equal fractions were probed with monoclonal anti-DnaK antibody from StressGen Biotech. Lane 1, 50 ng of purified DnaK from StressGen Biotech. Lane 2, Sucrose gradient purified reconstituted 30S particle. Lane 3, Sucrose gradient purified and Centricon 100 treated reconstituted 30S particle.

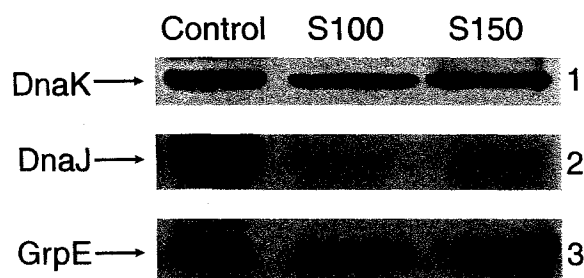


Figure 2. Western Blot Analysis of *E. coli* extracts for the DnaK Chaperone System.

Control, 0.5 μ g of purified protein (DnaK, DnaJ or GrpE) purchased from StressGen Biotech.

S100, 20 μ g S100 extract; S150, 20 μ g S150 extract. Lane 1, membrane probed with

monoclonal anti-DnaK antibody. Lane 2, membrane probed with polyclonal anti-DnaJ

antibody. Lane 3, membrane probed with polyclonal anti-GrpE antibody. All antibodies

were purchased from StressGen Biotech. S100 prepared as reported in (Maki et al., 2002).

S150 prepared as reported by (Nierhaus, 1990).

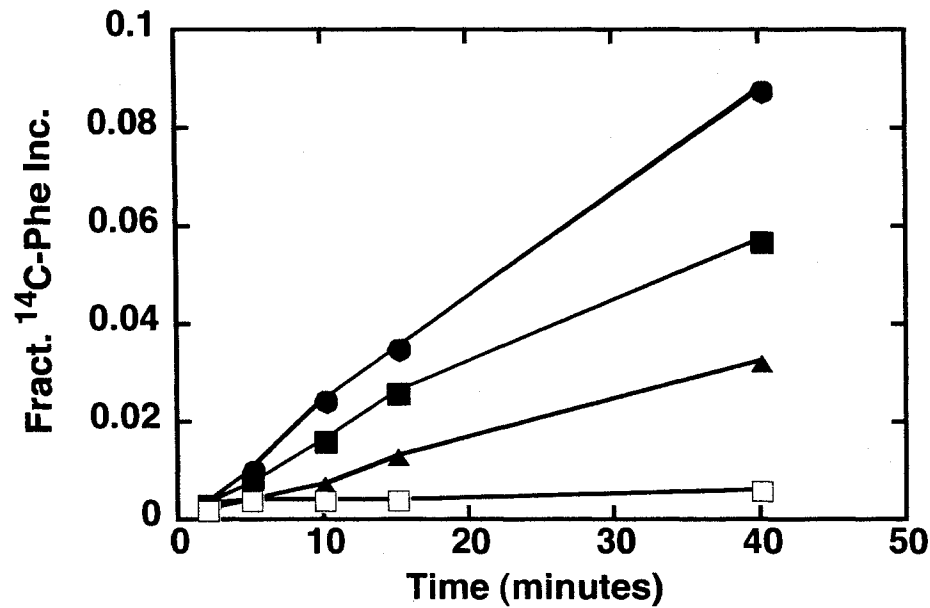


Figure 3. Polyphenylalanine synthesis by purified *in vitro* reconstituted *E. coli* 30S particles.

Reconstitutions were performed under the conditions of Alix and Nierhaus (2003).

Polyphenylalanine generated by purified 30S particles reconstituted under normal, high temperature (42°C) conditions (●), compared to natural 50S subunits alone (□), or particles reconstituted at low temperatures (20°, 21S) (▲) or as for the 21S reconstitution but in the presence of the DnaK chaperone system (■). Chaperone conditions were similar to those of Maki et al, 2002 (16S rRNA:DnaK:DnaJ:GrpE 1:1:1:2 with 1mM ATP). The polyphenylalanine synthesis was carried out, essentially as described (Southworth et al., 2002), by incubating 30S particles and 50S subunits (0.3 μM) with poly-uridine (0.35 mg/ml), ¹⁴C Phe-tRNA^{Phe}, His-tagged EF-G (0.3 μM), His-tagged EF-Tu (2 μM), GTP (1.4 mM), phosphoenolpyruvate (3.5 mM) and pyruvate kinase (14 μg/ml). Reactions were carried out in 80 mM HEPES pH 7.6, 13 mM MgCl₂ and 100 mM KCl.

**CHAPTER 4. ASSOCIATION OF THE DnaK CHAPERONE SYSTEM
COMPONENTS WITH *ESCHERICHIA COLI*
SMALL RIBOSOMAL SUBUNIT INTERMEDIATES**

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Abstract

It has been previously shown that the DnaK chaperone system can facilitate small subunit ribosome assembly *in vitro* at an otherwise non-permissive temperature. In this study, the DnaK chaperone system components' interaction with the assembling 30S subunit intermediates are further investigated using semi-quantitative western blot analysis, affinity chromatography and stabilization assays. Our results indicate that the interactions of DnaK, DnaJ and GrpE with 30S subunit intermediates resemble those observed in DnaK chaperone system/protein substrate interactions. Furthermore, DnaK interacts *in vivo* with both precursor and mature 16S rRNA as well as small subunit ribosomal proteins S3 and S4, suggesting that DnaK binds these 30S assembly intermediates *in vivo*. Finally, DnaK is capable of stabilizing the otherwise unstable intermediate RI, a critical step in small subunit formation. These data suggest that the DnaK chaperone system acts during assembly of the 30S subunit in a manner consistent with its well-documented role in protein folding.

Keywords: Ribosome, 30S subunit, DnaK, hsp70, assembly.

Introduction

Ribosomes are complex macromolecular assemblages. The *Escherichia coli* (*E. coli*) ribosome has a sedimentation coefficient of 70S and is composed of two subunits: the 30S (small) and 50S (large) subunits. *In vitro* assembly of 30S subunits has been achieved utilizing 16S ribosomal RNA (rRNA) and various sources of ribosomal proteins (r-proteins) including Total Protein from the 30S subunit (TP30)(Traub and Nomura, 1968), individually purified natural proteins (Held et al., 1973), and individually purified recombinant proteins (Culver and Noller, 1999). The assembly process follows an ordered pathway in which 16S rRNA and subsets of the proteins assemble in distinct steps (Held et al., 1974; Mizushima and Nomura, 1970). The first subset of proteins is termed primary binding proteins because they bind independently to naked 16S rRNA. Secondary binding proteins require the prior association of at least one primary binding protein in order to bind, and tertiary binding proteins require the binding of at least one primary binding protein and one secondary binding protein before they associate with the ribonucleoprotein particle (RNP) (Held et al., 1974; Mizushima and Nomura, 1970).

30S subunit assembly can be dissected into a series of discrete steps. At low temperature, a stall in assembly occurs, and an intermediate particle (Reconstitution Intermediate, RI) that sediments at 21S and consists of 16S rRNA and a subset of the small subunit r-proteins (the primary and secondary binding proteins) results (Traub and Nomura, 1968). *In vitro* formed RI particles are relatively unstable (Held and Nomura 1973; Maki et al., 2002), however upon heat-treatment, a more stable particle, RI*, forms. RI* sediments at 26S, has essentially the same molecular components as RI, but it is capable of associating with the tertiary binding proteins to form functional 30S subunits. Particles similar to RI have been observed *in vivo* and can be chased into functional 30S subunits (Alix and Guerin,

1993; Guthrie et al., 1969; Lindahl, 1975; Nashimoto et al., 1971), suggesting the *in vitro* and *in vivo* assembly pathways are similar.

The *in vitro* requirement for heat activation suggested that factors exist *in vivo* to assist in this assembly process. Utilizing S100, a crude *E. coli* extract, and low temperature *in vitro* reconstitutions, assembly factors were identified associated with 30S subunit intermediates (Maki et al., 2002). The DnaK chaperone system (DnaK, DnaJ, GrpE and ATP) was sufficient to facilitate *in vitro* 30S subunit assembly at low temperature. The particles resulting from this treatment co-sediment with and have the same protein complement as 30S subunits, and were functional as determined by a tRNA binding assay. In further studies, polyphenylalanine synthesis was achieved using DnaK chaperone system-formed 30S subunits (Maki et al., 2003). Additionally, it was determined that DnaK interacts with a subset of small subunit ribosomal proteins *in vitro* (Maki et al., 2002).

The DnaK chaperone system, an hsp70 chaperone system, has a well-characterized role in protein folding (Bukau and Horwich, 1998). In the protein folding cycle, DnaJ binds substrate, recruiting it to DnaK in the ATP bound state. DnaJ accelerates DnaK's hydrolysis of ATP to ADP, effecting a conformational change in DnaK and concomitant tight binding of substrate. Next, GrpE binds in the ATPase domain of DnaK and causes nucleotide exchange and substrate release. DnaK is once again in the ATP bound state and the folding cycle can begin again. Thus, the properties of association for each chaperone component with protein substrates have been relatively well defined.

In this work, the interaction of the DnaK chaperone system with small ribosomal subunit assembly intermediates was explored using sucrose gradient sedimentation, semi-quantitative western blot analysis, stabilization assays, and affinity chromatography. These studies allow the chaperone reaction cycle in 30S subunit assembly to be investigated and then compared with the cycle described for protein folding. Our results demonstrate that the

chaperones interact with 30S subunit intermediates in a manner that is consistent with their known roles in protein folding. Also, an interaction between DnaK and 30S subunit components *in vivo* was observed using DnaK affinity chromatography. Finally, it is also shown that DnaK is capable of stabilizing the *in vitro* RI particle much as heat does in the formation of RI*. Taken together, these studies begin to reveal aspects of a functional capacity for the DnaK chaperone system in 30S subunit assembly.

Results

General approach

To understand the interaction of the DnaK chaperone system with assembling 30S subunits, *in vitro* reconstitution was performed with purified chaperone components. In this manner, the relationship between assembling 30S subunits and DnaK, DnaJ and GrpE was assessed. These studies were pursued to determine if and how the DnaK chaperone system components interact with 30S subunit intermediates and whether the chaperone system was working in a manner similar to that observed in its protein-folding role. Previous work has demonstrated the importance of purification of chaperone-treated 30S subunit intermediates for analysis (Alix and Nierhaus, 2003; Maki et al., 2002; Maki et al., 2003). In this work, purification of intermediate particles via sucrose gradient sedimentation coupled with semi-quantitative western blot analysis was employed to monitor association of chaperone components with *in vitro* formed 30S intermediates under various conditions. This allows association of chaperone components with the pre-30S subunits to be assessed but does not allow binding constants to be determined. In brief, 16S rRNA, TP30 and chaperone components were incubated together at 15°C, sucrose-gradient purified, equalized according to RNA content, and subjected to subsequent analysis.

DnaJ/30S intermediate interactions

In protein folding, DnaJ recruits substrate to DnaK and accelerates the rate of ATP hydrolysis to lock the DnaK in the DnaK-ADP-substrate conformation (Wittung-Stafshede). In previous work, it was shown that DnaJ (in the context of S100 extract) did not stably associate with small subunit assembly intermediates (Maki et al., 2002). However, DnaJ was present in the extract and thus could assume a more transient association as observed in the protein folding cycle. Here, using a purified set of chaperone components, DnaJ association with assembly intermediates can be observed under various conditions (Figure 1a II). In the absence of other components, DnaJ is found associated with the intermediate RNP both in the presence and absence of ATP (Figure 1a II, lanes 4 and 5). When both DnaK and DnaJ are added to the intermediate particle, association of DnaJ is observed again in the presence or absence of ATP (Figure 1a II, lanes 6 and 7). However, in the presence of the complete chaperone system, the addition of ATP appears to have an effect on DnaJ association (Figure 1a II, compare lanes 8 and 9). Association of DnaJ with the intermediate is observed in the presence of DnaK and GrpE, without added ATP (Figure 1a II, lane 8). However, in the presence of ATP, significantly less DnaJ is found associated with the assembly intermediate (Figure 1a II, lane 9). These results are slightly different from those observed using S100 extract (Maki et al., 2002) and suggest that in high functional concentrations and in the absence of competition from extract components, DnaJ can indeed be found associated with 30S subunit intermediates. However, under the conditions that are most permissive for 30S subunit assembly, the abundance of DnaJ associated with the intermediate is reduced. These results suggest that DnaJ may play a role in pre-binding the intermediate and aiding in DnaK association, very much like the role of DnaJ in protein folding.

DnaK/30S intermediate interactions

DnaK is the central player in the hsp70 protein folding cycle. It binds hydrophobic patches of un- or misfolded proteins and facilitates their proper folding. ATP hydrolysis

enables DnaK's tight binding to substrate, and the conformational change that occurs when ADP is exchanged allows it to release substrate (McCarty et al., 1995). In this study using purified components, DnaK was found associated with 30S subunit assembly intermediates under various conditions (Figure 1a-c). DnaK association with the 30S intermediate RNP is observed in the presence of DnaJ (Figure 1a I, lane 6) and in the presence of DnaJ and GrpE (Figure 1a I, lane 8). In both cases, the addition of ATP leads to a reduction in the amount of bound DnaK (Figure 1a I, lanes 7 and 9), with this result being most pronounced in the presence of the full chaperone system (Figure 1a I, lane 9). It was also demonstrated that DnaK binds to the pre-30S RNP in the absence of its co-chaperones either with or without ATP (Figure 1b, lanes 2 and 4). Virtually no bound DnaK is detected in the presence of DnaK, GrpE and 1 mM ATP (Figure 1b, lane 7). In contrast, when DnaJ is also present, bound DnaK can be detected (Figure 1b, lane 6). One possible explanation for this observation is DnaJ-facilitated rebinding of DnaK to the assembly intermediate. Overall, these results suggest that DnaK interacts with the 30S subunit intermediate in a manner that is consistent with its interaction with protein substrates.

To further investigate the effect of ATP on DnaK binding, ATP and temperature titrations were performed on particles formed in the presence of the entire DnaK chaperone system. At 15°C, the amount of DnaK bound to assembling intermediates rapidly decreased with a corresponding increase in the concentration of ATP from 0 to 1 mM (Figure 1c, lanes 2-4). This trend was also observed when particles were formed under these conditions but incubated at 42°C (Figure 1c, lanes 5-7), although the initial amount of DnaK bound appeared to be much lower than that seen at 15°C, suggesting a more dynamic interaction at higher temperature. These results suggest DnaK binding to 30S subunit assembly intermediates is influenced by not only its co-chaperones but also by temperature and ATP concentration. These observations may have marked functional implications since it was

previously shown that association of DnaK with 30S particles inhibits their function (Maki et al., 2003).

GrpE/30S intermediate interactions

GrpE's role in the protein folding cycle is aiding DnaK in nucleotide exchange and substrate release (Harrison, 2003). In this analysis, DnaK, DnaJ and GrpE were incubated with assembly intermediates at 15°C in the presence and absence of ATP. GrpE was found associated with intermediates in the absence of ATP (Figure 1a III, lane 8) however the presence of ATP results in a dramatic decrease in detectable GrpE (Figure 1a III, lane 9). This is consistent with GrpE's release with DnaK at the completion of a folding cycle. However, these results are slightly different than those found previously with *E. coli* S100 extract (Maki et al., 2002), where GrpE was found associated with the RNP in the presence of DnaK, DnaJ and ATP. The slight discrepancy can be attributed to the use of individually purified DnaK chaperone system components and a significantly higher concentration of ATP, resulting in more complete release of GrpE.

Generally, these results describing the DnaK chaperone system association with 30S subunit intermediates appear to be consistent with what is known about the interactions of the DnaK chaperone system components with substrates during the protein folding cycle. Although this analysis does not delve into the mechanism, the association results follow the convention of the previously elucidated roles of DnaK and its co-chaperones DnaJ and GrpE in protein folding (Bukau and Horwich, 1998). Therefore, it appears that the reaction binding cycle may be similar between protein substrates and this RNP.

DnaK interacts with small subunit ribosomal components in vivo.

Next, a role for DnaK in 30S subunit assembly *in vivo* was addressed. Previous *in vivo* studies using a *dnaK* mutant allele (*dnaK756*) suggested that the absence of DnaK resulted in ribosome biogenesis defects in *E. coli* (Alix and Guerin, 1993). However, these

studies did not reveal a direct role in 30S subunit assembly for DnaK *in vivo*. In the yeast *Saccharomyces cerevisiae* (Harnpicharnchai et al., 2001), an affinity chromatographic approach using a protein shown to bind pre-ribosomal particles has proven powerful as a tool for characterizing and purifying factors that are associated with ribosomal assembly intermediates *in vivo*. Here a related approach was used to determine if DnaK bound 30S subunit intermediates *in vivo*. An inducible, tagged version of DnaK was used to allow overexpression of DnaK within wild type cells and subsequent affinity chromatography of DnaK and associated cellular components. The bound components were eluted by a potassium chloride gradient (330 mM to 1M KCl), a high salt ATP wash, and finally removal of DnaK and bound components from the column. Initially, the resulting fractions were assayed by native gel electrophoresis for the presence of large RNAs to determine if DnaK associates with ribosomal particles *in vivo*. 16S rRNA was found in all samples eluted, and the amount of RNA detected varied in different samples (Figure 2a, lanes 1-7). This observation suggests that the RNA might be present in different complexes that display differential affinity for DnaK. 23S rRNA was released with DnaK from the column (Figure 2a, lane 7). The presence of 23S rRNA with the eluted DnaK protein might be attributed to DnaK's association with the 50S subunit portion of the 70S ribosome. However, since 23S rRNA is not observed in the other fractions, association with the 50S subunit alone cannot account for the presence of 16S rRNA in various column fractions. These results indicate that DnaK indeed interacts with rRNA *in vivo*.

Given that DnaK appears to be associating with precursor 30S subunits *in vitro* (Figure 1), we next investigated whether the 16S rRNA associated with DnaK *in vivo* is mature at its 5' end. Primer extension analysis was used to determine the extent of 16S rRNA processing. In all lanes, the 5' end of the 16S rRNA can be clearly seen as a very strong band in the primer extension gel (Figure 2b). Interestingly, an unusually large

proportion of precursor 16S rRNA was also observed in the samples eluted from the column (Figure 2b, lanes 1-7) as compared with a control extension (Figure 2b, lane C). Very little unprocessed 16S rRNA was observed in the control 16S rRNA isolated from wild type 30S subunits (Figure 2b, lane C). When the amount of precursor 16S rRNA is expressed as a ratio of precursor to processed 16S rRNA, the ratio for control unmodified 16S rRNA is only 0.01, whereas fractions eluted from the DnaK affinity column range from 0.15 to 0.38. The presence of 16S rRNA with a pre-5' end suggests that DnaK is interacting *in vivo* with immature 16S rRNA, possibly in the context of the 30S subunit precursor.

To assess whether small subunit ribosomal proteins are also found associated with DnaK *in vivo*, elution of S4 and S3 from the DnaK column was monitored. In the absence of RNA, these proteins were previously shown to bind a DnaK column with different affinities. These proteins were also chosen because of their different properties in 30S subunit assembly. S4 is a primary binding protein, and thus can be expected in early stage assembling small subunits, while S3 is a tertiary binding protein and will be present only near the end of 30S subunit assembly. S3 was found in earlier eluting fractions (Figure 3, lanes 1 and 2), while S4 was detected in both early and late fractions (Figure 3, lanes 1-7). The presence of S4 in the later eluting fractions, where S3 is absent, raises the possibility that DnaK is associating with partially assembled 30S subunits. This would indicate a role for DnaK in 30S subunit assembly *in vivo*. This assertion is consistent with data using a mutant allele of *dnaK* where under non-permissive conditions, 30S subunit assembly is impaired (Alix and Guerin, 1993). Taken together, these affinity chromatography results suggest that DnaK may interact with 30S subunit precursors *in vivo*, as premature 16S rRNA and small subunit ribosomal proteins are co-eluting from the DnaK column. Also, it appears that DnaK could have altered affinity for more mature 30S subunits than for early assembly intermediates, suggesting differential interaction with these particles.

DnaK chaperone system components stabilize assembly intermediates.

While the work presented here indicates that the DnaK chaperone system, and most notably DnaK itself, interacts with 30S subunit assembly intermediates, the consequences of such interactions are still not well understood. It has been observed that *in vitro*-formed RI particles are unstable (Held and Nomura, 1973; Maki et al., 2002). However, the heat-activated form of this intermediate, RI* appears to be more stable. Thus, this stabilization of the intermediate can be used to monitor a transition during 30S subunit assembly and to determine if the DnaK chaperone system stabilizes the RI particle in a manner similar to heat treatment. RI particles were formed and their stability was tested using different heat and chaperone conditions. As expected, the protein complement of RI (Figure 4, lane 3) is reduced relative to that observed for heat-formed RI* (Δ RI*) (Figure 4, lane 4). The protein complement of the particles treated at low temperature with DnaK alone or DnaK and ATP (Figure 4, lanes 5 and 6) more closely resembles that of Δ RI*, suggesting DnaK or DnaK and ATP are capable of stabilizing the RNP in a manner analogous to heat treatment. Due to the small and similar size (approximately 10-23 kDa) of primary and secondary binding proteins, resolution of the individual proteins by SDS-PAGE is not ideal. However, S4 and S7, two of the largest components of RI and RI* can be resolved relatively easily and are prominent in Δ RI* and in the DnaK treated particles. The stabilization of pre-30S particle by DnaK could be a crucial part of the mechanism by which 30S subunits are assembled under otherwise non-permissive conditions. Stabilizing properly folded intermediates could increase the overall efficiency of 30S subunit formation, thus suggesting a significant role for DnaK in this process.

Discussion

Utilizing a variety of techniques including semi-quantitative western blot analysis, primer extension, affinity chromatography and stability assays, the interactions of the DnaK

chaperone system components and the forming 30S subunit of the *E. coli* ribosome were analyzed. The *E. coli* hsp70 chaperone system components were all observed to interact with pre-30S particles and the interactions appear, at least at this level of analysis, to be similar to those observed between the chaperone components and protein folding substrates. This suggests that there may be similarities between the mechanism by which DnaK folds proteins and facilitates 30S subunit assembly. Additionally, data was presented that suggests DnaK can act to stabilize 30S subunit intermediates and associate with pre-30S particles *in vivo*. These findings suggest a significant role for the DnaK chaperone system in 30S subunit formation.

For the first time, DnaJ was found associated with 30S subunit assembly intermediates in the presence and absence of DnaK and ATP. However, in the presence of DnaK, GrpE and ATP, less DnaJ was associated with the assembly intermediate (Figure 1a II). These results are consistent with our previous data (Maki et al., 2002); when crude *E. coli* extract (S100) was used, DnaJ was not found associated with the RNP. Under these conditions, all of the chaperone components and ATP were present and thus no DnaJ association was detected. DnaK was found to bind 30S assembly RNPs in the presence of DnaJ or DnaJ and GrpE. Under both conditions, the amount of bound DnaK detected decreased in the presence of ATP. Virtually no DnaK was observed in association with the assembly intermediate when DnaJ, GrpE and ATP were present as well (Figure 1a I, 1b, and 1c). Also, the amount of DnaK bound to the pre-30S RNP was decreased by both increased temperature and concentration of ATP (Figure 1c). Finally, GrpE bound the RNP in the presence of DnaK and DnaJ, but its association was greatly reduced in the presence of ATP (Figure 1a III). These results demonstrate that all of the DnaK chaperone system components are capable of interacting with 30S subunit assembly intermediates and that the

observed interactions follow a pattern that is reminiscent of the interaction of these same components when folding protein substrates.

DnaK has been shown to interact with 70S ribosomes and to play a role in ribosome assembly *in vivo* (Alix and Guerin, 1993; Alix and Nierhaus, 2003). In recent work (Hage et al., 2004), the DnaK chaperone system was found to facilitate the assembly of *in vivo* formed 21S (RI) intermediates. Additional *in vivo* work has shown that overexpression of small subunit ribosomal proteins S4 and S8 can rescue the temperature sensitive phenotype of the mutant dnaK allele, *dnaK756* (Maki et al., 2002). In the studies presented here, DnaK's *in vivo* interaction with 30S subunit components was explored utilizing affinity chromatography coupled with native gel electrophoresis, primer extension, and western blot analysis. Affinity chromatography of tagged DnaK revealed that pre-16S rRNA, the primary binding protein S4, and to a significantly less extent tertiary binding protein S3, associate with DnaK *in vivo* (Figures 2b and 3). These data raise the intriguing possibility that DnaK may assist in the early stages of 30S subunit assembly *in vivo*. It is possible that DnaK could be recruiting primary binding proteins (such as S4) that bind near the 5' end of 16S rRNA early in assembly. Additionally, the involvement of precursor 16S rRNA with DnaK hints at the possibility of co-transcriptional formation of the 30S subunit *in vivo*, a likelihood that has generated much interest.

The temptation might be to speculate that DnaK is interacting with ribosomal small subunit proteins simply by virtue of DnaK binding regions within these proteins. However, when an algorithm designed to predict DnaK binding sites (Rudiger et al., 1997) was used, none of the ribosomal proteins were identified as ideal DnaK substrates (unpublished results). However, this should not come as a surprise, as the characteristic DnaK binding region is hydrophobic, and none of the small subunit ribosomal proteins are overly hydrophobic. The interaction of chaperones with RNA is not as prevalent in the literature as

their interaction with proteins, but it is not unprecedented. For example, DnaK has been shown to interact with 5S rRNA by Northwestern analysis (Okada et al., 2000). Zimmer and colleagues propose that chaperones may assist in RNA folding and demonstrated that human hsp70's RNA binding activity is influenced by its co-chaperones (Zimmer et al., 2001). Additionally, GroEL (a chaperonin) has been implicated in 9S rRNA processing, perhaps via regulation of RNase E (Sohlberg et al., 1993). Thus it would not be unprecedented if DnaK interacted with 16S rRNA as part of the 30S subunit intermediate RNP and not just with individual ribosomal small subunit proteins or 16S rRNA.

The stabilization of 30S subunit assembly intermediates by the DnaK chaperone system suggests that DnaK facilitates 30S subunit assembly in this manner. RI particles are inherently unstable, but when heat-treated, they form RI*, a particle that sediments at 26S, exhibits increased stability, and is competent for further assembly into functional 30S subunits. Using sucrose gradient sedimentation, molecular sieving filters, and SDS-PAGE, this stabilization, previously only observed with heat treatment at 42°C, was achieved at low temperature with DnaK (Figure 4) or the entire DnaK chaperone system (data not shown). Thus it appears that DnaK is acting at this stage of 30S subunit assembly.

These results suggest that through recruitment of the proper components and stabilization of assembly intermediates, the DnaK chaperone system is able to assist in the assembly of 30 subunits. It appears that the DnaK components may interact with not only small subunit proteins, but also the 16S rRNA-containing RNPs in this function. The *in vivo* binding of precursor 16S rRNA in this context also brings up the intriguing possibility that the DnaK chaperone system could aid in the co-transcriptional assembly of ribosomal small subunits *in vivo*.

Materials and Methods

In vitro reconstitution, sucrose gradient purification, and western blot analysis.

In vitro reconstitution and sucrose gradient purification of particles performed as described by Culver and Noller (1999; 2000b). In all reconstitution experiments, the final buffer concentration was 80 mM K⁺-Hepes pH 7.6, 20 mM MgCl₂, 330 mM KCl, and 0.01% Nikkol. The concentration of 16S rRNA was 0.4 μM, while the chaperone components were present at concentrations of: 0.4 μM DnaK and DnaJ, 0.8 μM GrpE, and 1 mM ATP unless otherwise noted. TP30 concentration was 1.6 μM in Figure 1a and 2 μM in 1b and 1c. Recombinant proteins were present at a concentration of 3.6 μM in Figure 4. The concentrations used are based on optimization experiments for individual protein preparations.

For TP30 experiments, 16S rRNA was heated at 42°C for 20 minutes, and then cooled on ice for 15 minutes. TP30 and chaperone components were added, and the particles were incubated at 15°C for 2 hours. Particles were then subjected to sucrose gradient sedimentation as described (Maki et al., 2002). The particles were collected from gradients, and absorbance readings at 260 nm were taken. Equal amounts according to RNA absorbance were directly precipitated with ethanol (2.5 X) for Figure 1a and b. In Figure 1c, equal amounts were loaded on centricon 100s (Amicon, Millipore Corp. Bedford, MA). Particles were concentrated then washed twice with 2.5 ml of Protein Storage Buffer (PSB; 1 M KCl, 20 mM MgCl₂, and 80 mM K⁺-Hepes pH 7.6), prior to being ethanol precipitated. Western blot analysis was then performed as previously described (Maki et al., 2002).

In the recombinant protein experiment, 16S rRNA was heated at 42°C for 20 minutes, and then cooled on ice for 15 minutes. Primary binding proteins were then added and incubated 20 minutes on ice. Secondary binding proteins were added, and particles were again subjected to 20-minute incubation on ice. Chaperone components were added and particles were incubated at 15°C for 30 minutes. After sucrose gradient purification, equal amounts (according to OD₂₆₀) were loaded on centricon 100s and particles were treated as

described above. Tricine SDS PAGE (Schagger and von Jagow, 1987) and silver-staining (Marshall, 1984) were then performed.

Anti-DnaK, -GrpE, -DnaJ, and secondary antibodies were from StressGen (Victoria, BC, Canada). The ECL western blot analysis kit was purchased from Amersham-Pharmacia (Piscataway, NJ) and used as directed.

DnaK affinity chromatography and analysis

ER2566 cells in which chitin-binding domain tagged DnaK had been overexpressed (Maki et al., 2002) were resuspended in a magnesium-free buffer (330 mM KCl, 20 mM K⁺Hepes 7.6, 2.5 mM EDTA, 6 mM BME). The cells were then sonicated and the resulting supernatant was loaded on a 3-ml chitin column pre-equilibrated in the same buffer. The column was then washed with two column volumes of this buffer. All of the following column washes were performed with buffers that include the components listed above but also 10 mM MgCl₂ and increasing amounts of KCl: one column volume each at 500 mM KCl, 750 mM KCl, and 1 M KCl. ATP was then added to a concentration of 100 μ M, and the column was rocked in the cold room for 30 minutes. The flow-through was collected, and the column was washed with one column volume of 1 M KCl. Finally, the DnaK was cleaved from the column with 40 mM DTT overnight and then collected. RNA was extracted from samples as described (Culver and Noller, 2000a) for native gel electrophoresis on a tris composite gel (Dahlberg and Peacock, 1971) and primer extension (Merryman and Noller, 1998; Moazed et al., 1986), whereas western analysis was performed on the samples after ethanol precipitation (see above).

Tris composite gel

The Tris composite gel was 3% acrylamide (37.5:1), 1X TBE, and 0.5% agarose.

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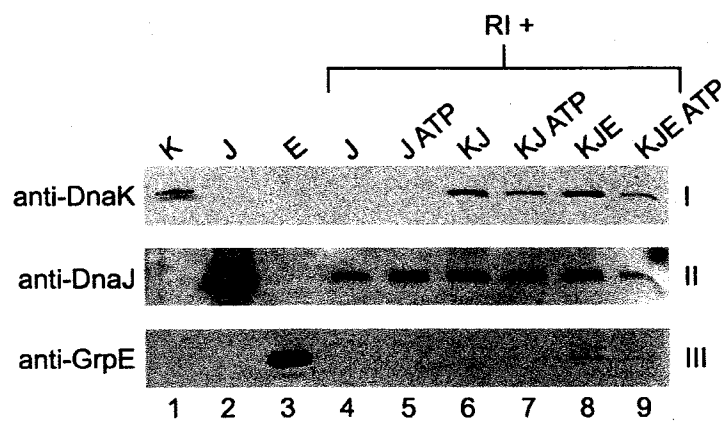
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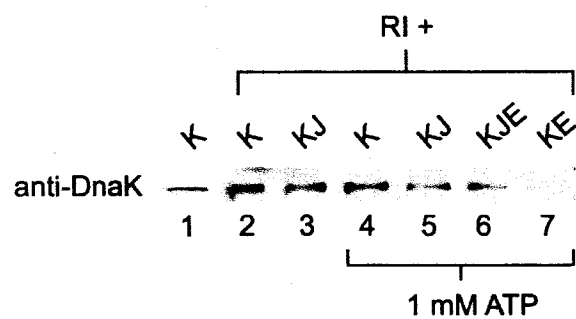
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Figure 1

a.



b.



c.

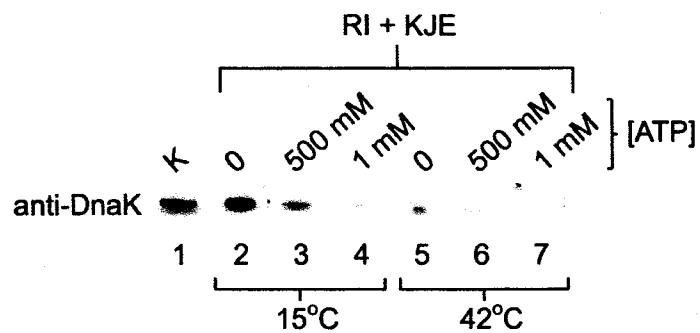


Figure 1: The DnaK chaperone system components interact with purified 30S subunit assembly intermediates.

a. Western blot analysis for DnaK chaperone components interactions with sucrose gradient purified in vitro reconstituted 30S subunit intermediate particles.

Particles were formed at 15°C using 16S rRNA and TP30. The membrane was probed with polyclonal anti-DnaJ (II) and anti-GrpE (III) antibodies, then stripped and re-probed with monoclonal anti-DnaK (I) antibodies. Lanes 1-3 are control loadings of purified DnaK (K), DnaJ (J), and GrpE (E), respectively. Lanes 4-9, RI particles incubated with the indicated DnaK chaperone components.

b. Western blot analysis for DnaK chaperone components interactions with sucrose gradient purified in vitro reconstituted 30S subunit intermediate particles.

Particles were formed at 15°C using 16S rRNA and TP30. The membrane was probed with monoclonal anti-DnaK antibodies. Lane 1, purified DnaK (K). Lanes 2-7, RI particles incubated with indicated DnaK chaperone system components and ATP where noted.

c. Western blot analysis of the effect of temperature and ATP on DnaK association with sucrose gradient purified in vitro reconstituted 30S subunit intermediate particles.

Particles were formed at 15°C or 42°C using 16S rRNA and TP30. The membrane was probed with monoclonal anti-DnaK antibodies. Lane 1, purified DnaK (K). Lanes 2-7, RI particles incubated with indicated DnaK chaperone system components and ATP in concentrations noted by lane.

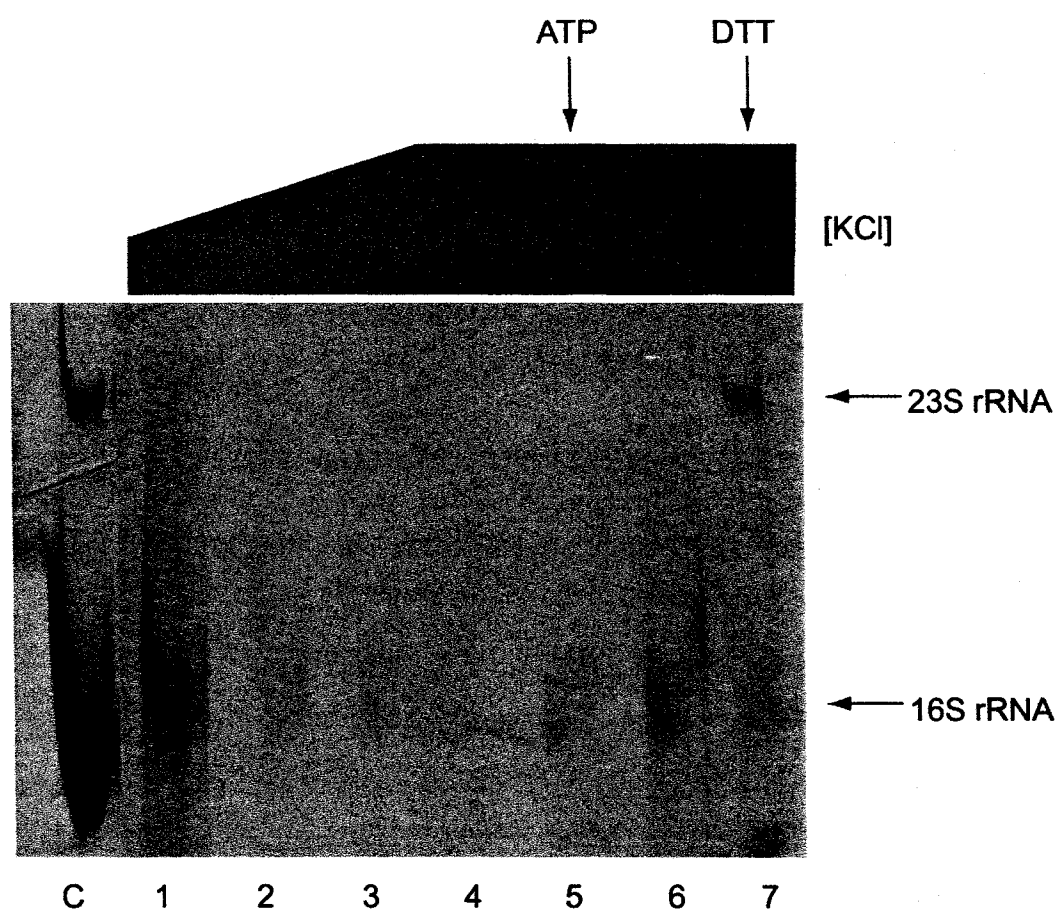


Figure 2: DnaK interacts with 16S rRNA *in vivo*.

a. Native gel electrophoresis of RNA eluted from a DnaK affinity column.

Lanes labeled ATP and DTT correspond to samples collected after incubation with ATP and DTT, respectively. Lane C (control), purified 16S and 23S rRNA. Lanes 1-7 representative fractions that were eluted with the given KCl concentration after initial binding of extract to column and subsequent washes. Lane 1, 330 mM KCl. Lane 2, 500 mM KCl. Lane 3, 750 mM KCl. Lane 4, 1 M KCl. Lane 5, 100 μM ATP flow through. Lane 6, 1 M KCl. Lane 7, 40 mM DTT flow through.

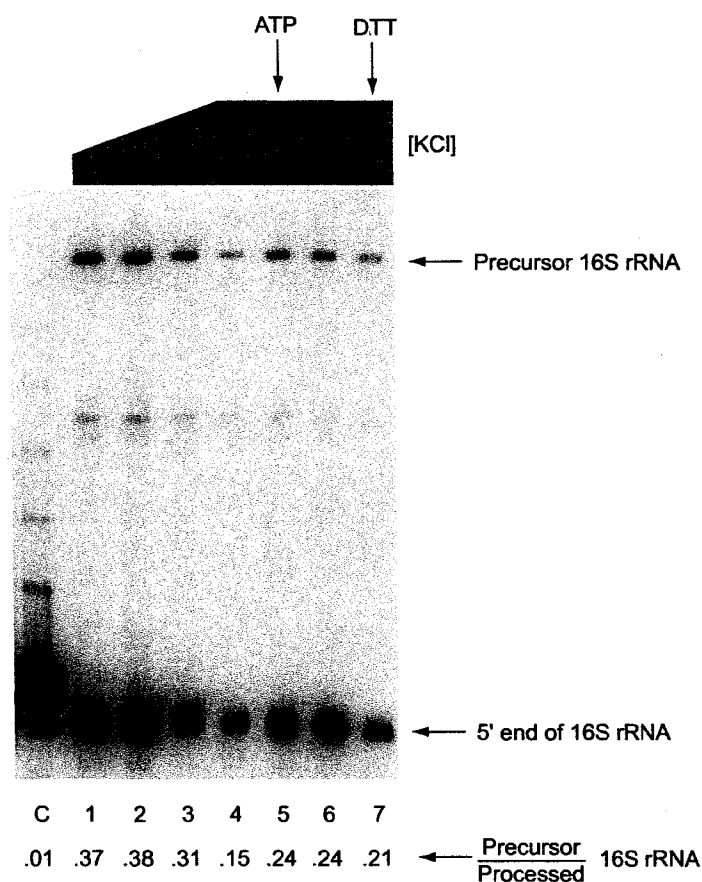


Figure 2: DnaK interacts with 16S rRNA *in vivo*.

b. Primer extension analysis of RNA samples eluted from the DnaK affinity column.

The positions of the 5' end of mature 16S rRNA and the precursor 16S rRNA are indicated. Lanes labeled ATP and DTT correspond to samples collected after incubation with ATP and DTT, respectively. Lane C (control) purified 16S rRNA. Samples were prepared as described in part A. Lane 1, 330 mM KCl; Lane 2, 500 mM KCl; Lane 3, 750 mM KCl; Lane 4, 1 M KCl; Lane 5, 100 μ M ATP flow through; Lane 6, 1 M KCl; Lane 7, 40 mM DTT flow through. The ratio of precursor to processed 16S rRNA as determined using NIH Image is denoted for each sample.

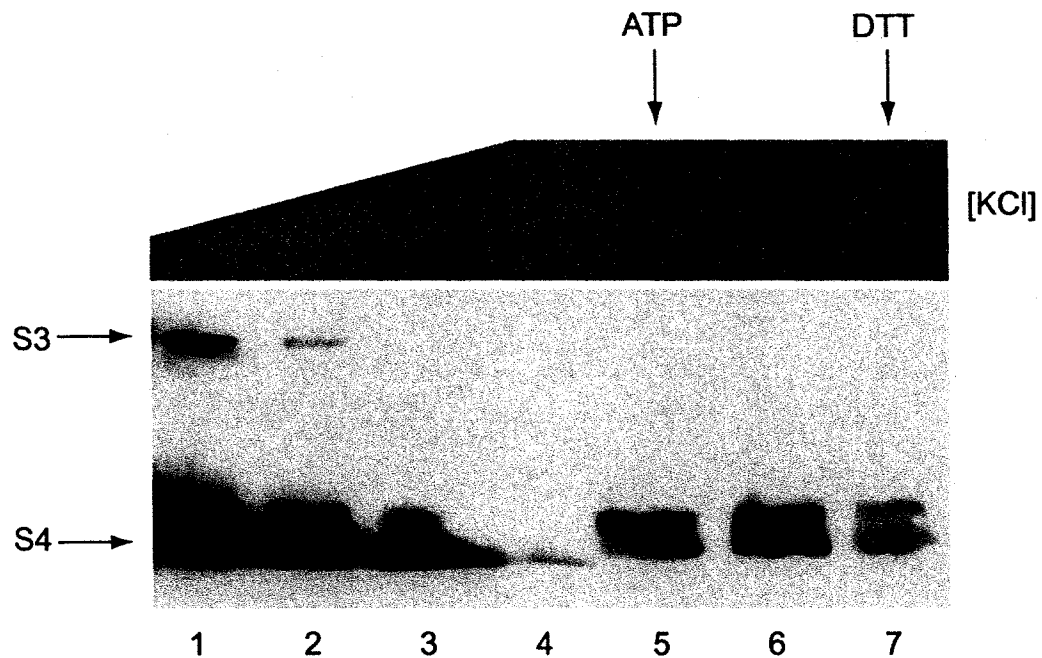


Figure 3: DnaK differentially interacts with small subunit ribosomal proteins S4 and S3.

Western blot analysis of proteins eluted from the DnaK affinity column.

The membrane was probed with monoclonal anti-S3 and anti-S4 antibodies. The positions of S3 and S4 are noted. Lanes labeled ATP and DTT correspond to samples collected after incubation with ATP and DTT, respectively. Samples were prepared as described in Figure 2.

Lane 1, 330 mM KCl; Lane 2, 500 mM KCl; Lane 3, 750 mM KCl; Lane 4, 1 M KCl; Lane 5, 100 μ M ATP flow through; Lane 6, 1 M KCl; Lane 7, 40 mM DTT flow through.

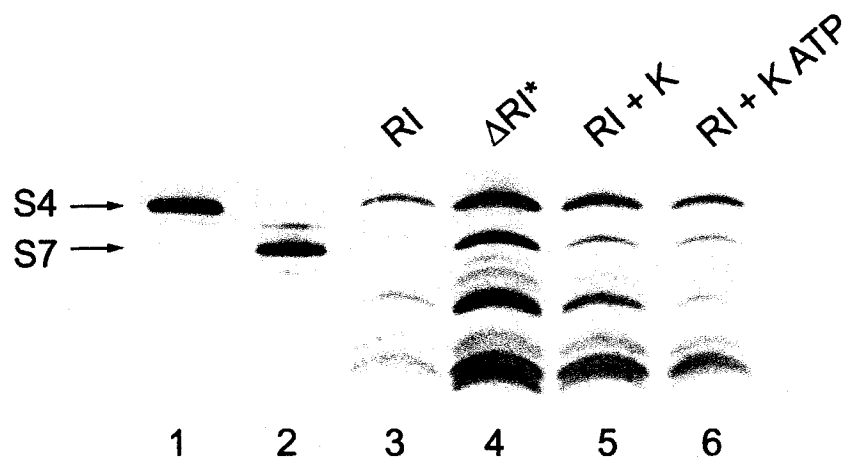


Figure 4: DnaK stabilizes the 30S subunit assembly intermediate RI.

Tricine SDS PAGE analysis of in vitro reconstituted sucrose gradient purified particles. The positions of S4 and S7 are indicated. All reconstitutions performed at low temperature except Δ RI*, which was incubated at 42°C. Lane 1, purified S4. Lane 2, purified S7. Lane 3, Reconstitution Intermediate. Lane 4, Δ RI*, RI treated at 42°C. Lane 5, RI incubated with DnaK. Lane 6, RI incubated with DnaK and ATP.

CHAPTER 5. GENERAL CONCLUSIONS

Summary

In this body of work, several scientific discoveries have been made. In Chapter 2, my first manuscript, a role for DnaK in ribosome assembly was demonstrated. DnaK and GrpE were both found stably associated with assembling small ribosomal subunits *in vitro*. The purified DnaK chaperone system was able to circumvent the heat requirement of *in vitro* reconstitution, assisting in the formation of 30S subunits with the proper sedimentation profile, protein complement and tRNA binding activity. DnaK was also found to bind small subunit proteins, both purified and from whole cell lysate, suggesting a true *in vivo* interaction with ribosomal components. Further bolstering the argument for *in vivo* relevance, the overexpression of two small subunit ribosomal proteins that DnaK binds partially rescued a temperature sensitive *dnaK* mutant. The overexpression of S4 in this strain was shown to partially rescue its ribosome defect. These results indicate DnaK is a ribosome assembly factor and there is a link between two highly conserved systems: ribosomes and hsp70s (Maki et al., 2002). In spite of being highly conserved, *dnaK* is not essential in the cell (Paek and Walker 1987), so it follows that there must be additional factors *in vivo* that are capable of assisting in ribosome assembly. Our lab is eager to identify these additional factors and follow a similar characterization of their function in ribosome assembly. Perhaps trigger factor, a chaperone that binds the ribosome, will be the chaperone that helps ribosome assembly in the absence of DnaK. The GroEL/ES system, a cylindrical chaperonin that actively folds its substrates, could be another candidate, given that it can assist in the survival of the double knockout $\Delta tig \Delta dnaK$ at 30°C (Vorderwulbecke et al., 2004). However, *in vitro* reconstitution of 30S subunits at low temperature with the assistance of the GroEL/ES system was attempted and unsuccessful (Maki, unpublished results).

Alix and colleagues previously suggested that DnaK and ribosome biogenesis are linked (Alix and Guerin, 1993). In 2003, Alix and Nierhaus explored the facilitation of 30S subunit association by the DnaK chaperone system. In this work, the authors determined that the presence of the DnaK chaperone system did not augment polyphenylalanine synthesis of forming 30S subunits *in vitro*. They also examined ribosomes from the *dnaK* null strain grown under permissive conditions and found that the thermostability of these ribosomes was comparable to wild type ribosomes. From these results, and not having repeated any of our experiments, they declared that our results inconsistent with theirs (Alix and Nierhaus, 2003).

In Chapter 3, my second manuscript, these issues were addressed. To begin, we revealed the two major differences between the polyphenylalanine synthesis assay performed by Alix and Nierhaus and the assay performed in the Culver and Green labs. The first difference was the lack of particle purification by Alix and Nierhaus. After reconstitution, particles must be sucrose gradient purified before any functional assays are performed (Maki et al., 2003). Otherwise, DnaK will remain bound to the assembling RNP and interfere with its functional abilities. The second discrepancy in the experiment was the use of S150 extract. Normally, the enzymes necessary for polyphenylalanine synthesis *in vitro* are provided by addition of crude *E. coli* extract, either S150 or S100. In this case, Alix and Nierhaus had gone to great lengths to assure the control particles were devoid of DnaK chaperone components. Unfortunately, adding S150 to the reaction equalized the control particles with the DnaK chaperone treated particles, as S150 contains the entire DnaK chaperone system (Maki et al., 2003). Utilizing sucrose gradient purified particles and a defined polyphenylalanine system, polyphenylalanine synthesis was demonstrated with *in vitro* low temperature, DnaK chaperone system-formed 30S subunits (Maki et al., 2003).

In the studies of ribosomes from the *dnaK* null strain grown at permissive temperature vs. wild type strains, Alix and Nierhaus focused on the thermostability of

ribosomes. Those isolated from the null strain behave similarly to those from the wild type cells. This is not surprising, as DnaK is likely not the only ribosome assembly factor in *E. coli*. *dnaK* is not essential in the cell (Paek and Walker, 1987), and as such we never claimed it was the only factor capable of assisting in ribosome assembly. In fact, we suggested that there were more such factors to be discovered for this very reason (Maki et al., 2002). In a bizarre turn of events, Alix's laboratory has recently shown that *in vivo* formed 21S intermediates were assembled more quickly in the presence of the DnaK chaperone system (Hage et al., 2004). This result verifies our *in vitro* assembly studies by demonstrating that 21S particles are true 30S assembly intermediates *in vivo*. Furthermore, it reveals that the DnaK chaperone system facilitates complete assembly of *in vivo* formed 30S assembly intermediates.

In Chapter 4, my final manuscript, the DnaK chaperone system component's association *in vitro* with pre-30S particles was dissected. The interactions were very similar to DnaK chaperone system interactions in protein substrate folding. DnaK is likely working in ribosome assembly via a mechanism similar to that used in protein folding. DnaK was associated *in vivo* with pre-processed 16S rRNA and S4, suggesting a role for DnaK in the early stages of 30S subunit assembly. DnaK could assist in recruiting early assembly proteins that bind near the 5' end of 16S rRNA to allow cotranscriptional formation of 30S subunits. This would not be an unprecedented role for a protein like DnaK. As discussed in Chapter 1, the 60 kDa chaperonin from *Sulfolobus solfataricus* bound pre-processed 16S rRNA and primary binding proteins as well (Ruggero et al., 1998).

The *in vivo* association of DnaK with pre-16S rRNA is very intriguing. This interaction could be possible because of DnaK's propensity to bind AU motifs (Zimmer et al., 2001). In pre-16S rRNA, the sequence near the mature 5' end is 5'-UUUUAAAUUGAA-3' where the underlined A is the beginning of the mature 5' end. This

could be an ideal site for DnaK to bind, effecting more efficient and correct 30S subunit assembly. In a related example, mammalian hsp70 binds A + U rich elements (AREs) and has been postulated to regulate ARE-directed mRNA turnover (Wilson et al., 2001). Additionally, hsp70 homologues bind polysomes *in vivo* (Beck and DeMaio, 1994; Pfund et al., 1998). It follows that hsp70s from across phylogeny may have a hand in transcription and/or translation. At this point, we have only a few small clues concerning these roles, but undoubtedly this field is going to expand rapidly in the next decade or so.

Broad implications of our work

In conclusion, a better understanding of *in vitro* 30S ribosomal subunit assembly could have much more broad consequences, perhaps leading scientists to much more efficient ways to treat bacterial infection. With the rampant overuse of antibiotics and the resulting multi-resistance strains of bacteria, we will soon need a more direct way to treat bacterial infection. If the intricacies of prokaryotic ribosome assembly were known, tools could be developed to target these infections at the level of bacterial ribosome biogenesis. Moreover, with the assistance of assembly factors, *in vitro* ribosome assembly could lead to the mass production of small peptides and proteins used as novel therapeutics. This sort of protein production could change the way the pharmaceutical industry operates.

Future directions

RI→RI transition, DnaK vs. heat*

My labmate Kristi Holmes has been using chemical modification and primer extension analysis to study the effect of DnaK on RI particles, a project at the intersection of our research interests. Using the DnaK footprint on the assembly intermediates as a point of reference, she has been able to determine changes in reactivity due to the action of DnaK. Thus far, Holmes has found a number of the same protections and enhancements observed in the RI→RI* transition catalyzed by heat (Holmes, unpublished results). This approach could

also be used in future studies of the association behavior of the co-chaperones to verify what we have seen by Western blot analysis and expand our understanding of the entire process.

DnaK-16S rRNA interactions

The binding of 16S rRNA by DnaK needs to be further explored. DnaK association with 16S rRNA has been demonstrated by sucrose gradient sedimentation in both the presence and absence of ATP (Maki, unpublished results). Additionally, peptide competition experiments have been performed that demonstrate 16S rRNA is bound robustly by DnaK in the presence of a large excess of peptide substrate. This suggests DnaK binds 16S rRNA in its ATPase domain, as previously determined with other RNA examples (Henics et al., 1999; Zimmer et al., 2001). Control experiments still need to be performed. The first and most important of these is the testing of other large RNAs' interaction with DnaK to verify that this DnaK binding is specific.

Identification of additional assembly factors

Since the *dnaK* null strain is viable (Paek and Walker, 1987), additional ribosome assembly factors must exist. These factors will be screened for in much the same way we identified DnaK. S100 extract will be prepared from the null strain and employed in low temperature reconstitution experiments. It has been demonstrated that S100 from the null strain is capable of the RI→30S conversion (Narayanaswamy, unpublished results), so any non-ribosomal proteins that associate with the assembling particles should be easily identified. Following identification, a similar course of action will be taken with the factor(s) (Maki et al., 2002). Another approach to this line of experimentation would be to prepare S100 extract from wild type cells and immunodeplete it of DnaK by treatment with an excess of DnaK IgG-Sepharose (Goasduff and Cederbaum, 2000). Once additional assembly factor(s) are known, it would be interesting and informative to use all factors at low temperature but with physiological salt conditions to determine whether the artificial *in vitro*

reconstitution conditions could be replaced with a more relevant mock *in vivo* environment. This sort of optimization could completely change the way we dissect 30S subunit assembly and allow us to apply our knowledge to the *in vivo* system more readily.

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